ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF ARTEMETHER AND LUMEFANTRINE IN POWDER FOR ORAL SUSPENSION DOSAGE FORM BY RP-HPLC

A Dissertation Submitted to

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In Partial fulfillment for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL ANALYSIS

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF ARTEMETHER AND LUMEFANTRINE IN POWDER FOR ORAL SUSPENSION DOSAGE FORM BY RP-HPLC ",** submitted by the student bearing **Reg. No: 261530204** to "**The Tamil Nadu Dr.M.G.R.Medical University – Chennai**" , in partial fulfillment for the award of Degree of **Master of Pharmacy** in **Pharmaceutical Analysis** was evaluated by us during the examination held on……..…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation "**ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF ARTEMETHER AND LUMEFANTRINE IN POWDER FOR ORAL SUSPENSION DOSAGE FORM BY RP-HPLC",** submitted to **"The Tamil Nadu Dr.M.G.R.Medical University**", Chennai, was carried out by **Mrs. A. EZHILVANI [Reg.No: 261530204],** for the Partial fulfillment of degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS** under my guidance and supervision during the academic year 2016-2017

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DECLARATION

The work presented in this dissertation entitled "**ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF ARTEMETHER AND LUMEFANTRINE IN POWDER FOR ORAL SUSPENSION DOSAGE FORM BY RP-HPLC**" was carried out by me, under the direct supervision of **Dr.I.CAROLIN NIMILA, M.Pharm., Ph.D.,** Assistant Professer of Pharmaceutical Analysis, J.K.K.Nattaraja College of Pharmacy, Kumarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

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Dedicated to
Almighty
My Beloved Parents,
Husband,
&
My Family Members

LIST OF ABBRAVATIONS

API	Active pharmaceutical Ingredient
CGLP	Current Good Laboratories Practices
CGMP	Current Good Manufactures Practices
CV	Co-efficient of Variation
°c	Degree centigrade
FTIR	Fourier Transmission Infra Red
ICH	International Conference on Harmonization of
K	Capacity factor
LOD	Limit of detection
LOQ	Limit of Quantitation
mg	Milligram
mcg	microgram
mL	Milliliter
mg/mL	Milligram per milliliter
N	Plate number
NLT	Not less than
NMT	Not more than
nm	Nanometer
PDA	Photo Diode Array
RI	Refractive Index
RP-HPLC	Reverse phase High Performance Liquid Chromatography
RS	Resolution
RSD	Relative Standard Deviation
RT	Retention time
SD	Standard Deviation

T	Tailing factor
USP	United States Pharmacopoeia
UV	Ultra violet
v/v	Volume per volume
µg	Micro gram
µg/mL	Microgram per Milliliter
ppm	Parts per million
Fig	Figure
ART	Artemether
LUM	Lumefantrine

TABLE OF CONTENTS

1. INTRODUCTION

The pharmaceutical analysis is a branch of chemistry, which involves the series of process for the identification, determination, quantization, and purification. This is mainly used for the separation of the components from the mixture and for the determination of the structure of the compounds. The different pharmaceutical agents are as follows:

1. Plants
2. Microorganisms
3. Minerals
4. Synthetic compounds

Based upon the determination type, there are mainly two types of analytical methods. They are as follows:

1. **Qualitative analysis**: This method is used for the identification of the chemical compounds.
2. **Quantitative analysis**: This method is used for the determination of the amount of the sample.

Types of Analytical Methods

Analytical methods are mainly of the following two types:

1. **Classical methods**:
    1. Gravimetry: The weight of the sample is determined after the precipitation.

2. Titrimetry: The volume of the solution is determined after the reaction such as neutralization, complex formation, precipitate formation, and oxidation and reduction.
3. Volumetry: The volume of the gas evolved by the reaction is determined.

2. **Instrumental methods**:

1. Electrochemical methods: Used for the measurement of the current, voltage, or resistance.

Examples: Conductometry: Measurement of the conductance.

Potentiometry: Measurement of the potential.

Coulometry: Measurement of the current.

Voltametry: Measurement of the current at specified voltage.

2. Optical methods: Based upon the measurement of radiation absorbed or emitted

Absorption methods : Visible, Ultraviolet (UV), Infrared (IR), Atomic Absorption Spectroscopy (AAS)

Emission methods: Plasma emission spectroscopy, flame spectroscopy, and fluorimetry.

3. Chromatography: paper, High Pressure Liquid Chromatography (HPLC), Gas Chromatography (GC), ion exchange, Thin Layer Chromatography (TLC), and column chromatography.

4. Thermal methods: Differential Thermal Analysis (DTA), Thermogravimetric (TG), and Differential Scanning Calorimetry (DSC).
5. Other methods: X-ray diffractometry, radioactive methods, mass spectrometry, refractometry and polarimetry.

Pharmaceutical analysis deals not only with medicaments (drugs and formulations), but also with their precursors i.e. with the raw material whose degree of purity, which in turn decides the quality of medicaments. The quality of a drug is determined, after establishing its authenticity, which is carried by testing its purity and the quality of the pure substance in the drug and its formulations.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC):

High-performance liquid chromatography is a chromatographic technique used to separate the components in a mixture, to identify each component, and to quantify each component .The method involves a liquid sample being passed over a solid adsorbent material packed into a column using a flow of liquid solvent. Each analyte in the sample interacts slightly differently with the adsorbent material, thus retarding the flow of the analytes. If the interaction is weak and the analytes flow off the column in a short amount of time, and if the interaction is strong, then the elution time is long.

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases**.** (Sharma B.K) [1]

The schematic representation of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/V is, photodiode array (PDA) or Refractive index (RI).

CHROMATOGRAPHY AND ITS TYPES

HPLC techniques are classified on the following types:

- Based on the modes of chromatography (based on the polarity of stationary and mobile phase):

Normal phase mode: Stationary phase is polar e.g., silica gel and mobile phase is non-polar.

Reverse phase mode: Stationary phase is non-polar and mobile phase is polar.

- In normal phase mode, non-polar compounds travel faster and are eluted first. This is because of less affinity between solute and stationary phase. Polar compounds are retained longer time in the column because of more affinity towards stationary phase and take more time to elute.

- In reverse phase mode, polar compounds get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature and not retained for a longer time and eluted faster.

Chromatography is a family of analytical chemistry techniques for the separation of mixtures. It involves passing the sample, a mixture that contains the analyte, in the "mobile phase", often in a stream of solvent, through the "stationary phase." The stationary phase retards the passage of the components of the sample. When components pass through the system at different rates they become separated in time, like runners in a marathon. Ideally, each component has a characteristic time of passage through the system. This is called its "retention time."

A physical separation method in which the components of a mixture are separated by differences in their distribution between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves through it in a definite direction. The substances must interact with the stationary phase to be retained and separated by it.

A chromatograph takes a chemical mixture carried by liquid or gas and separates it into its component parts as a result of differential distributions of the solutes as they flow around or over a stationary liquid or solid phase. Various techniques for the separation of complex mixtures rely on the differential affinities of substances for a gas or liquid mobile medium and for a stationary adsorbing medium through which they pass; such as paper, gelatin, or magnesium silicate gel.

Analytical chromatography is used to determine the identity and concentration of molecules in a mixture. Preparative chromatography is used to purify larger quantities of a molecular species.

Based on the principle of separation:

1. **Adsorption chromatography**: Separation of compounds based on the difference in affinity of compounds towards stationary phase. Most widely used stationary phase is unmodified silica which allows high efficiency and high permeability. The functional group responsible for adsorption is silanol group which reacts with sample solutes by hydrogen bonding.
2. **Ion exchange chromatography**: Separation of compounds based on ion exchange of functional groups. In this ion exchange, resins are used to separate a mixture of similar charged ions. The retention of the ions on the column depends on the ionic strength and PH of the mobile phase. Types of ion exchangers include the following:
    1. **Polystyrene resins**: These allow cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity.
    2. **Cellulose and dextran ion exchangers (gels)**: These possess larger pore sizes and low charge densities making them suitable for protein separation.
    3. **Controlled-pore glass or porous silica**: In general, ion exchangers favour the binding of ions of higher charge and smaller radius.

3. **Ion pair chromatography**: In this reverse phase, column is converted temporarily into ion exchange column using compounds such as pentane or hexane or heptanes or octane with sulphonic acid sodium salt, tetra methyl or ethyl ammonium hydroxide.
4. **Size exclusion chromatography (gel permeation chromatography)**: Separation is based on the different molecular size compounds separated by using different gels.

   Example: Dextran, agarose, polyacrylamide gels. This technique is widely used for the determination of molecular weight of polysaccharides.

5. **Affinity chromatography**: Separation is based on the affinity of the sample with specific stationary phases.
6. **Chiral phase chromatography**: Separation of optical isomers using chiral stationary phases.

- Based on elution technique:
  1. **Isocratic separation**: Same mobile-phase combination is used throughout the process of separation.
  2. **Gradient separation**: Mobile-phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.
- Based on scale of operation:
  1. Analytical HPLC

     Example: Analysis of samples (μg).

2. Preparative HPLC

Example: Individual fractions of samples are analysed (mg). (A.H Beckett et.al)[2]

COMPONENTS OF HPLC SYSTEM:

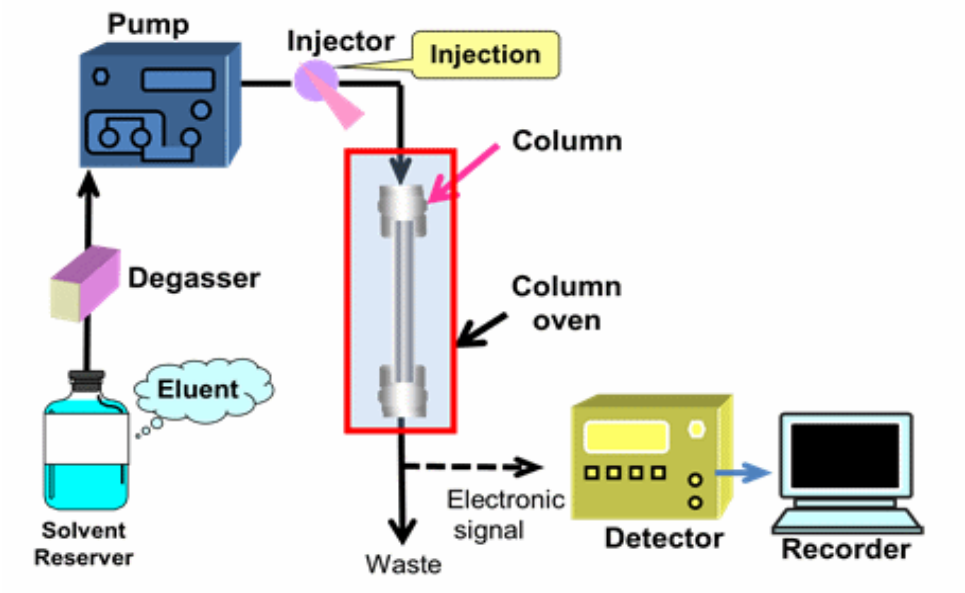
Pump

Pump generates a flow of elute from the solvent reservoir to the system. Most pumps used in current LC system generate the flow by back-and forth motion of a motor –driven piston. (Reciprocating pumps). Because of this piston motion, it produces "pulses". There have been large system improvements to reduce this pulsation and the recent pumps create much less pulse compared to the older ones. Recent analysis requires very high sensitivity to quantify a small amount of analytes, and thus even a minor change in the flow rate can influence the analysis. Therefore, the pumps required for the high sensitivity analysis needs to be highly precise.

Injector

An injector is placed next to the pump. The simplest method is to use a syringe, and the sample is introduced to the flow of eluent. Since the precision of LC measurement is largely affected by the reproducibility of sample injection, the design of injector is an important factor. The most widely used injection method is based on sampling loops. The use of auto sampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Figure1.1 Instumentation of HPLC



Column

The separation is performed inside the column; therefore, it can be said that the column is the heart of an LC system. The packing material generally used is silica or polymer gels. The eluent used for LC varies from acidic to basic solvents. Most column housing is made of stainless steel, since stainless is tolerant towards a large variety of solvents. However, for the analysis of some analytes such as biomolecules and ionic compounds, contact with metals is not desired, thus polyether ether ketone (PEEK) column housing is used instead.

Detector

Separation of analytes is performed inside the column, Whereas a detector is used to observe the obtained separation .The composition of the eluent is consistent when no analyte is present .While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences. This difference is monitored as a form of electronic signal.

On-line detectors

- ❖ Refractive index
- ❖ UV/Vis Fixed wave length
- ❖ UV/Vis variable wave length
- ❖ UV/Vis Diode array
- ❖ Fluorescence
- ❖ Conductivity
- ❖ Mass –Spectrometric (LC/MS)
- ❖ Evaporative light scattering

Off-line detector

- FTIR spiral disk monitor requires sample transfer on the germanium disk and following scanning in FTIR instrument.

Recorder

The change in eluent detected by a detector is in the form of electronic signal, and thus it is still not visible to our eyes. Nowadays, computer based data processor (integrator) is more common .There are software that are specifically designed for LC system. It provides not only data acquisition, but features like peak-fitting, base line correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However,

the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymers membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go though the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used on-line; it is more convenient and efficient.

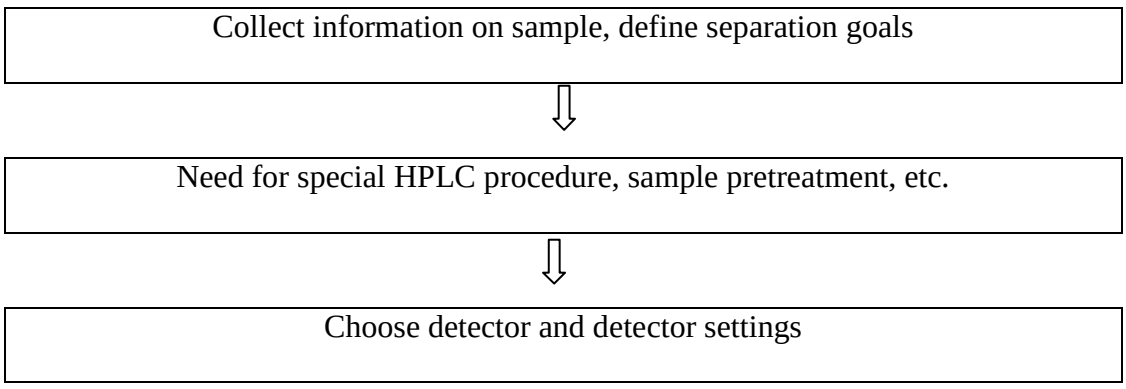
C**olumn heater**

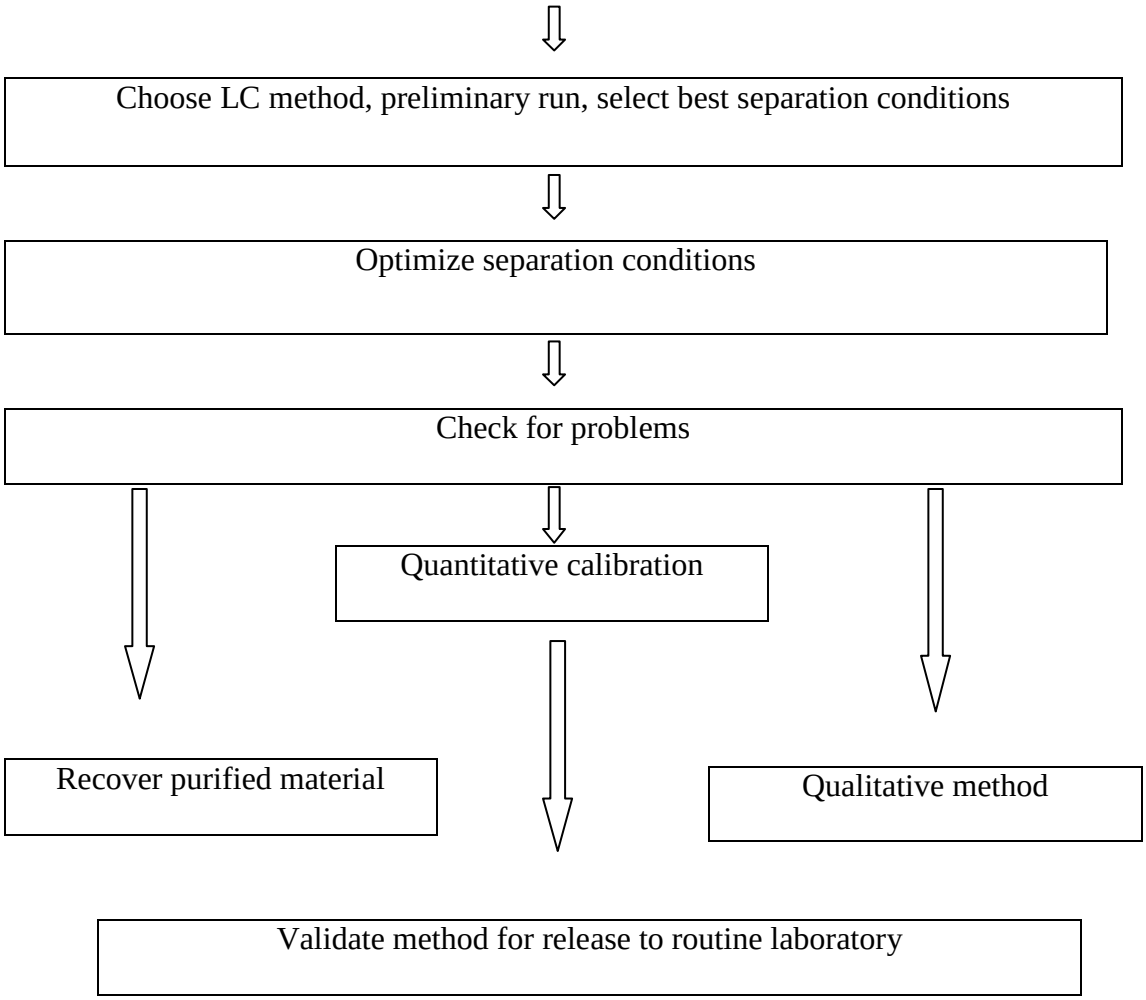
The LC separation is often largely influenced by the column temperature. Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50~80^{o}C). It is also important to keep stable temperature to obtained repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. The columns are generally kept inside the column oven (column heater).

(H.H Willard et.al, 1996)[3].

INTRODUCTION TO HPLC METHOD DEVELOPMENT

Method development has following steps:





A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are:

1. Careful sampling and sample preparation.
2. Appropriate choice of the column.
3. Choice of the operating conditions to obtain the adequate resolution of the mixture.
4. Reliable performance of the recording and data handling systems.

5. Suitable integration/peak height measurement technique.
6. The mode of calculation best suited for the purpose.
7. Validation of the development method.

(Synder et.al 1983)[4].

Careful sampling and sample preparation

Before beginning method development, it is need to review what is known about the sample in order to define the goals of separation. The sample related information that is important is summarized in below.

Number of compounds present
Chemical structure
Molecular weight of compounds
pk_a Values of compounds
UV spectra of compounds
Concentration range of compounds in samples of interest
Sample solubility

The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation.

Separation Goals

The goals of HPLC separation need to be specified clearly, which include:

- The use of HPLC to isolate purified sample components for spectral identification or quantitative analysis.
- It may be necessary to separate all degradants or impurities from a product for reliable content assay.
- In quantitative analysis, the required levels of accuracy and precision should be known (a precision of $\pm$ 1 to 2% is usually achievable).

- Whether a signal HPLC procedure is sufficient for a raw material or one or more different procedure are desired for formulations.
- When the number of samples for analysis at one time is greater than 10, a run time less than 20 minutes often will be important.

Sample preparation

Sample come in various forms:

- Solution ready for injection.
- Solutions that require dilution, buffering, addition of and internal standard or other volumetric manipulation.
- Solids must be dissolved or extracted.
- Samples that require pretreatment to remove interference and /or protect the column or equipment from damage.

Most samples for HPLC analysis require weighing and /or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvents is close to that of the mobile phase since this minimizes baseline upset and other problems. Some samples require a partial separation (pretreatment) prior to HPLC, because of need to remove interference, concentrate sample analyte or eliminate “column killers”.

The samples may be of two types, regular or special. The regular samples are typical mixtures of small molecules (<2000Da) that can be separated by normal starting conditions. Whereas special samples are better separated under customized conditions given below.

Choice of the column

The separation of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and partition modes, the

separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size of the ions generated by the sample molecules and the nature of ionisable group on the stationary phase. In case of size – exclusion chromatography the selection of the column is based on the molecular weight and size of the sample components. Selection of columns based on the method is briefly summarized in below.

Method /Description /Columns	**Preferred Method**
Reversed – Phase HPLC Uses water- organic mobile phase Columns: C_{18} (ODS), C8, Phenyl, trimethylsilyl (TMS), and cyano.	First choice for most samples, especially neutral or non-ionized compounds that dissolve in water-organic mixtures
Iron –pair HPLC Uses water – organic mobile phase, a buffer to control pH, and an ion –pair reagent Columns: C_{18} , C_8, Cyano	Acceptable choice for ionic or ionisable compounds, especially bases or cations.
Normal-phase HPLC Uses mixtures of organic solvents as mobile phase. Columns: cyano, diol, amino, silica	Good second choice when reserved-phase or ion-pair HPLC is ineffective; first choice for lipophilic samples that do not dissolve well in water-organic mixtures; first choice for mixtures of isomers and for preparative HPLC

Optimization of HPLC method

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts asymmetry, capacity factor, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest. Optimization of a method can follow either of two general approaches:

- Manual
- Computer driven

The manual approach involves varying one experimental variable at a time, while holding all other constant and recording changes in response. The variables might include flow rate, mobile or stationary phase composition, temperature, detection wavelength and p^H. This approach to system is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated method development, efficiency is optimized while experimental input is minimized. This approach reduce the time, energy and cost of all instrumental method development.

The various parameters that include to be optimized during method development are

A. Selection of mode of separation.
B. Selection of stationary phase.
C. Selection of mobile phase.
D. Selection of detector.

Selection of mode of separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

Selection of stationary phase

Selection of the column is the first and the most important step in method development. The appropriate choice of separation column indicates three different approaches.

- Selection of separation
- The particle size and nature of the column packing
- The physical parameters of the column i.e. the length and the diameter some of the important parameters considered while selecting chromatographic columns are
- Length and diameter of the column
- Packing material
- Shape of the particles
- Size of the particles
- % of carbon loading
- Pore volume
- Surface area
- Reproducibility and reliability
- End capping

In this case, the column selected had a particle size of 5µm and an internal diameter of 4.6mm.The column is selected depending on the nature of the solute and

the information about the analyte. Reversed phase mode of chromatography facilities a wide range of columns like dimethyl silane (C_2), butysilane (C_4), octylsilane (C_8), Octadecyslane (C_{18}), base deactivated silane **(**C_{18}), BDS phenyl, Cyanopropyl (CN), nitro, amino etc. silica based columns with different cross linking's in the increasing order of polarity are as follows:

<……..Non-polar…….moderately polar……..polar……..>

$C_{18} < C_8 < C_6 <$ Phenyl < Amino < Cyano < Silica

C_{18} was chosen for this study since it is most retentive one. The sample manipulation becomes easier with this type of column. Generally longer columns provide better separation due to higher the theoretical plate numbers. Columns with 5µm particle size give the best compromise of efficiency.

Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- Inaccurate plate number and resolution measurement
- Imprecise quantitation
- Degraded and undetected minor bands in the peaks tail
- Poor retention reproducibility

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

A column which gives separation of all the impurities and degradants from each other and from analyte peak and which is rugged for variation in mobile phase shall be selected.

Selection of mobile phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and from analyte peak.

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, solute-mobile phase, and mobile phase-stationary phase. For a given stationary phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation).Solvent polarity is the key word in the chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes. The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer
- pH of the buffer
- Mobile phase composition

Buffers if any and its strength

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4, NaH_2PO_4, and Na2HPO4.
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers-ammonium acetate, sodium acetate etc.
- Acetic acid buffers prepared using CH_2COOH.

The retention also depends on the molar strengths of the buffer-molar strength is increasingly proportional to retention times. The strength of the buffer can be increasing, if necessary to achieve the required separations. The solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength. The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight, silica may dissolve.

Mobile phase composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that fairly large amount of selectively can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and Acetonitrile. Experiments should be conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations of analyte peak. A mobile phase which gives separation of analyte peak

and which is rugged for variation of both aqueous and organic phase by at least $\pm$ 0.2% of the selected mobile phase composition should be used.

Selection of Detector

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, florescence, conductance, oxidation, reduction etc. The characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity facilitating trace analysis
- Negligible baseline noise to facilitate lower detection.
- Large linear dynamic range.
- Low dead volume.
- Inexpensive to purchase and operate.

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful. For the greatest sensitivity ƛmax should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wave lengths give greater selectivity.

Performance calculations

Carrying out system suitability experiment does the performance calculations. System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validations have been completed. The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation SD can be determined during validation.

System suitability might then require that retention times fall within a ± 3 SD range during routine performance of the method.

The USP (2000)[5] defines parameters that can be used to determine system suitability prior to analysis include plate number(n), tailing factor(T), resolution(Rs) and relative standard deviation (RSD) of peak height or peak area for respective injections.

The RSD of peak height or area of five injections of a standard solution is normally accepted as one of the standard criteria .For assay method of a major component, the RSD should typically be less than 1% for these five respective injections.

The plate number and / or tailing factor are used if the run contains only one peak. For chromatographic separations with more than one peak, such as an internal standard assay or an impurity method expected to contain many peaks, some measure of separations such as Rs is recommended. Reproducibility of t_R or k value for a specific compound also defines system performance.

The column performance can be defined in terms of column plate number. As the plate count is more the column is more efficient.

METHOD VALIDATION

The word "Validation'' means "Assessment'' of validity or action of proving effectiveness.

Definition

ICH[6] defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes.''

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Method needs to be validated or revalidated.

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics
- Whenever the method is changed, and the change is outside the original scope of the method.

Purpose of validation

- Enable the scientists to communication scientifically and effectively on technical matter.
- Setting the standards of evaluation procedures for checking compliance and taking remedial action.
- Economic: Reduction in cost associated with process sampling and testing.
- As quality of the product cannot always be assured by routine quality control because of testing of statistically insignificant number of samples.
- Retrospective validation is useful for trend comparison of results compliance to CGMP/CGLP.
- Closure interaction with pharmacopoeial forum to address analytical problems.
- International pharmacopoeial harmonization particularly in respect of impurities determination and their limits.

Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal

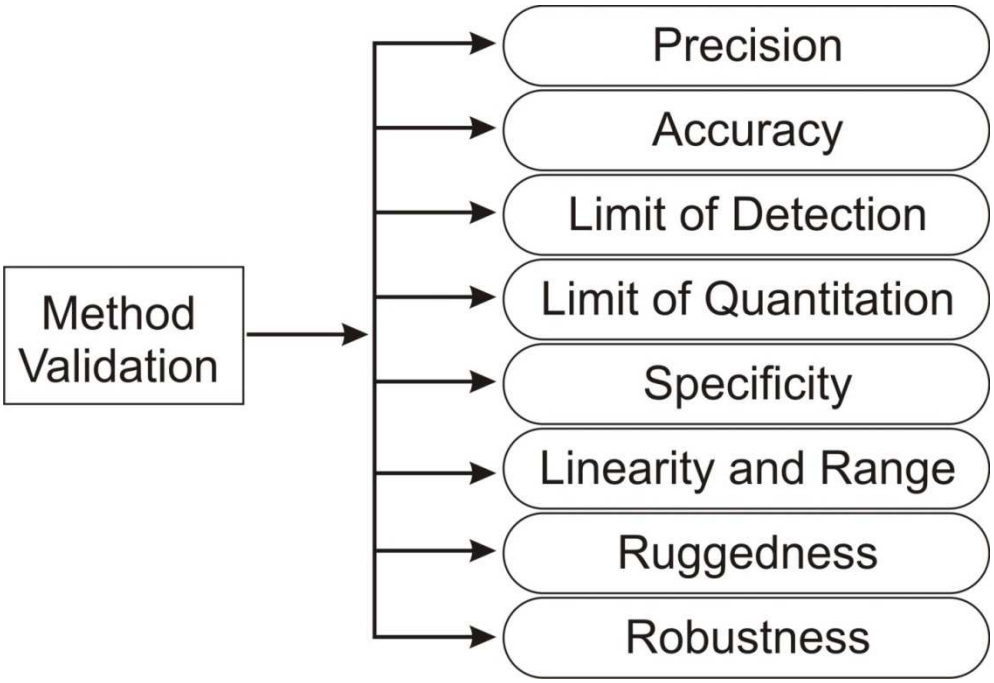
use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do''.

For method validation, these specifications are listed in USP chapter <1225>, and can be referred to as the "eight steps of method validation". As shown in figure below.

These terms are referred to as "analytical performance parameters'' Or sometimes as "analytical figures of merit.''

In response to this situation, one of the first harmonization projects taken up by the ICH was the development of a guideline on the "Validation of Analytical Methods" Definitions and Terminology. "ICH divided the "Validation characteristics" somewhat differently, as outlined in Figure below

The USP Eight steps of method validation



ICH Method Validation Parameters

Method validation parameters

The developed methods were validated by following steps:

A. **Accuracy**

It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. Spike and recovery studies are performed to measure accuracy; a known sample is added to the excipients and the actual drug value is compared to the value found by the assay. Accuracy is expressed as the bias or the % error between the observed value and the true value (assay value/actual value x 100 %.)

The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. The parameter provides information about the recovery of the drug from sample and effect of matrix, as recoveries are likely to be excessive as well as deficient.

Repeatability is the result of the method operating over a short time interval under the same conditions (or) is the % RSD of multiple determinations of a single sample in a single test run (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored (or) intermediate precision (also called inter-assay precision) measure the % RSD for multiple determinations of a single sample, controls and reagents analyzed in several assay runs in the same laboratory.

Reproducibility

It refers to the precision between laboratories usually in collaborative studies and not directly relevant to assay validation in a manufacturing facility. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval.

Specificity

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. It is not possible to demonstrate that an analytical procedure is specific for a particular analyte. In such case a combination of two or more analytical procedure is recommended to achieve the necessary level of discrimination. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures or tests.

In case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients and demonstrating that the assay is unaffected by the presence of these

extraneous materials. If the degradation product impurity standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g., pharmacopoeia method or other validated analytical procedure (independent procedure). These comparisons should include samples stored under relevant stress conditions (e.g. light, heat humidity, acid/base hydrolysis, oxidation.ect.)

Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to- noise ratio, usually two –or three-to-one. The ICH has recognized the signal-to-noise ratio convention, but also lists two other options to determine **LOD:** visual non-instrumental methods and a means of calculating the LOD. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. That is, as the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ.

Linearity

It is ability of an assay to obtain test results, which are directly proportional to the concentration of an analyte in the sample. The determination of linearity will Identify the range of the analytical assay. It can be measured as slope of the regression line and its variance or as the coefficient of determination (R^2) and correlation coefficient(R).

Range

Range is the interval between the upper and the lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. If the relationship between response and concentration is the linear, the range may be estimated by means of a calibration curve.

The range is normally expressed in the same units as the test results obtained by the method. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay the minimum specified range is from 80-120% of the target concentration. For an impurity test, the minimum range from the reporting level of each impurity, to 120% of the specification. (For toxic or more potent impurities, the range should be commensurate with the controlled level).

Ruggedness

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lot of reagents, different elapsed assay times, different assay temperatures different days, etc.

Robustness

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

System Suitability Test

System suitability test is commonly used to verity resolution, column efficiency and repeatability of the chromatographic system to ensure its adequacy for a particular analysis. According to the United States pharmacopoeia (USP) and the International Conference on Harmonization (ICH), SST is an integral part of many analytical procedure.

Primary SST parameters are most important as they indicate system specificity, precision and column stability. Other parameter include capacity factor (K) and signal to noise ratio(S/N) for impurity peaks.

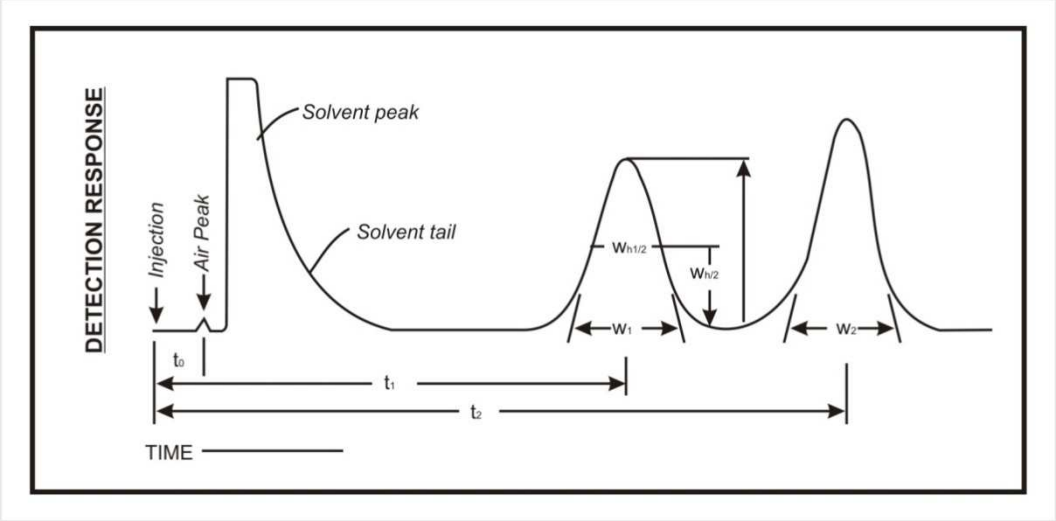
The USP chromatography general chapter states "System suitability test are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment,

electronics, analytical operations and samples to be analyzed constitute an integral system can be evaluated as such." (USP 38-NF 31, 621 – Chromatography) [5]

INTERPRETASTION OF CHROMATOGRAMS

Figure below represents a typical chromatographic separation of two substances, 1 and 2, where t_1 and t_2 are the respective retention times; and h, h/2, and $W_{h/2}$ are the height, the half – height, and the width at half-height, respectively, for peak1. W1 and W2 are the respective widths of peaks 1 and 2 at the base line. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.

Figure 1.2 Diagrammatic representation of Interpretation of chromatogram



Chromatography retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next.

Relative Retention times:

Relative retention time is calculated by the equation $R_r = t_2/t_1$

t_1 = Retention time of test.

t_2 = Retention time of reference substance, determined under identical experimental conditions on the same column.

Relative Retention:

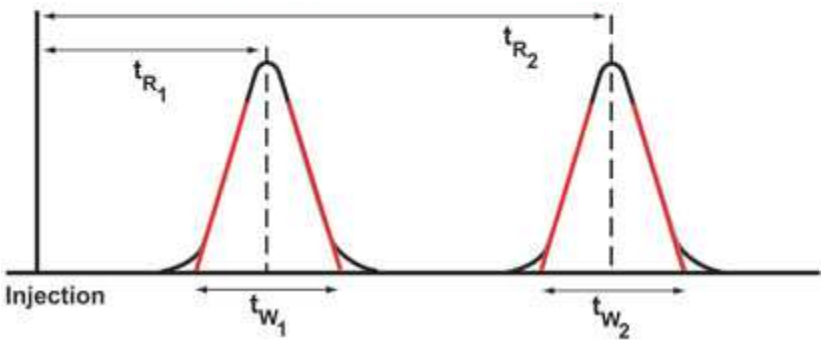
To calculate the relative retention (r) = $\frac{t_2 - t_M}{t_1 - t_M}$

Where t_M is the retention time of the non-retained marker.

Resolution

The resolution R is a function of column efficiency, N and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug.

Figure1.3 Resolution



R is determined by the equation:

$\mathbf{R}=\frac{2(t_2-t_1)}{W_{2+}W_1}$ Or

$$R = \frac{2(t_2 - t_2)}{1.70\,(W_{1,\,h/2} + W_{2,\,h/2})}$$

t_2 and t_1 are the retention times of the components.

W_2 and W_1 are the corresponding width at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the base line.

$W_{1h/2}$ and $W_{2h/2}$ are the corresponding peak width at half-height.

Resolution

$$R = \frac{1.18(t_{R2} - t_{R1})}{(W_{h1} + W_{h2})}$$

Where, $t_{R2} > t_{R1}$

$\mathbf{t_{R2}}$ and $\mathbf{t_{R1}}$ = Retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peak

$\mathbf{W_{h1}}$and $\mathbf{W_{h2}}$ = peak width at half height.

Theoretical Plates

Column efficiency also may be specified as system suitability requirements, especially if there is only one park of interest in the chromatograms. The number of the theoretical plates, N, is a measure of column efficiency. It is calculated by the equation.

$N = 16\,[t/w]^2$ or $N = 5.54\,[t/w^{1/2}]^2$

t = Retention time of the substance.

w = width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

$W^{1/2}$ = Peak width at half-height.

Precision:

Precision a measure of either degree of reproducibility or of repeatability is determined by making replicate injections of standard preparation and calculating relative standard deviation. Unless otherwise specified in the individual monograph, data from five replicate injections of the standard preparation are used to calculate the relative standard deviations (SR), if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

Relative Standard Deviation in percentage.

$$SR\ (\%) = \frac{100}{X}\left[\sqrt{\sum_{i=1}^{i=n}\frac{(Xi-\overline{x})\overline{2}}{N-1}}\right]$$

x = Arithmetic mean of the set.

xi = An individual measurement in a set of N measurements.

N= Number of individuals values

Tailing Factor (or) Symmetry factor

Tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing factor is pronounced (Fig 1). In some cases values less tan unity may be observed. As peak asymmetry increases, integration and hence precision becomes less reliable.

Tailing factor, $T = \frac{W0.05}{2F}$

W0.05 = Width of peak at 5% height.

F = Distance from the peak maximum to the leading edge of the peak, the distance is being measured at a point 5% of the peak height from baseline.

Capacity Factor (Mass distribution ratio):

Capacity factor k' of a sample component is a measure of the degree which that component is retained by the column relative to an unretained component

Capacity factor is $k' = \frac{(t_{r-t_0})}{t_0}$

t_r – is the elution time of retained component and

t_0 – is the elution time of the unretained sample.

Signal to Noise Ratio:

$S/N = \frac{2H}{H}$

Where,

H = Height of the peak corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height.

h = Range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

Peak to Valley ratio

The peak-to-valley ratio (p/v) may be employed as a system suitability requirement in a test for related substances when baseline separation between 2 peaks is not reached

$$P/v = \frac{H_p}{H_v}$$

H_p = Height above the extrapolated baseline of the minor peak,

H_v = Height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks. (ICH 2005)[6]

System Suitability Parameters and Recommendations:

Parameter	**Recommendation**
Capacity Factor (k')	The peak should be well-resolved from the other peaks and the void volume, generally k'>2.0
Repeatability	RSD, ≤1% for N≥5 is desirable
Relative retention	Not essential as long as the resolution is stated
Resolution	R_s of >2 between the peak of interest and the closed eluting
Tailing Factor (T)	T of ≤2
Theoretical Plates (N)	In general should be > 2000

STATISTICAL PARAMETERS

Linear regression:

Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is **y = mx + c**

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$\mathbf{m}= \frac{\mathbf{N}\sum \mathbf{xy}-(\sum \mathbf{x})(\sum \mathbf{y})}{\mathbf{N}\sum \mathbf{x2}-(\sum \mathbf{x})\mathbf{2}} \quad \text{and} \quad c= \frac{\mathbf{N}\sum \mathbf{y}\sum \mathbf{x2}-(\sum \mathbf{x})(\sum \mathbf{y})}{\mathbf{N}\sum \mathbf{x2}-(\sum \mathbf{x})\mathbf{2}}$$

Correlation coefficient:

When the changes in one variable are associated or followed by changes in the order it is called correlation. To establish whether there is a liner relationship between two variables x_1 and y_1, use Pearson's correlation coefficient r.

$$r=\frac{n\sum x1y1-\sum x1y1}{\{[n\sum x12-(\sum x1)2][n\sum y12-(\sum y1)2]\}1/2}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a define linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1, indicate negative correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

Standard deviation:

The standard deviation measures the spread f the data about the mean value. It is commonly used in statics as a measure of precision statics as a measure of Precision and is more meaningful than is the average and is expressed mathematically as.

$$S = \sqrt{\sum_{i=1}^{i=n} \frac{(Xi - \bar{x})}{N - 1}}$$

Where,

S is the standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the Denomination is N-1 or N

Σ = sum

X = observed values

$\bar{X}$= Mean or arithmetic average = ΣX/N

X - $\bar{X}$ = deviation of a value from the mean

N = Number of observations

Percentage relative standard deviation

It is also known as coefficient of variation CV. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

CV or % RSD = $\frac{S.D}{\bar{X}}$ x 100

Where, S.D = Standard deviation,

$\bar{X}$ = Mean or arthimetic average.

The variance is defined as S^2 and is more important in statistics than S itself. However, the latter is much more commonly used with chemical data.

Standard Error of mean:

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observation. It is mathematically expressed as

S.E = $\frac{S.D}{\sqrt{n}}$

Where, n = number of observations.

S.D = Standard Deviation

2. LITERATURE REVIEW

1. **R Arun et al**[13] described the development and validation of a simultaneous HPLC-UV method for the estimation of Artemether and Lumefantrine in fixed-dose combination tablets. The method showed to be linear (r2 > 0.999), precise (RSD <0.43%), accurate (recovery of 99.81% for Artemether and 99.54% for Lumefantrine), specific and robust. Three batches of Artemether and Lumefantrine tablets were assayed by the validated method. The Artemether contents in the tablets varied from 99.30 to 99.33%, while Lumefantrine contents were 99.65 to 99.66%.

2. **S Sharma et al**[14] had done estimation of Lumefantrine in pharmaceutical dosage forms, in that they described three simple, rapid, accurate, precise and cost-effective methods, I; formation and solving of simultaneous equation method, II; absorbance ratio method, III; Dual Wavelength Method have been developed for simultaneous estimation of Lumefantrine in tablet dosage form. Beer's law was obeyed in concentration range 5-35 µg/ml for Lumefantrine for all the proposed methods. The sampling wavelengths for methods I, II and III, selected for both the drugs were 252nm, 268nm and 296nm, on trial and error basis using0.01 N NaOH solutions as solvent. For methods I, II, III seven mixed standards solutions with concentration of Lumefantrine in the µg/ml of 5:35, 10:30, 15:25, 20:20, 25:15, 30:10 and 35:5, were prepared by diluting appropriate volumes of standard stock solutions for all proposed three methods. Results of analysis for six methods were tested and validated for various parameters according to ICH guidelines.

3. **Mohamed Aly Amin Ahmed Ibrahim et al** [15] developed a HPLC Method and did Validation for Determination of Lumefantrine in Pharmaceutical Dosage Forms, it described a simple, precise and rapid HPLC method was developed for estimation of Lumefantrine in pure and pharmaceutical dosage forms. The chromatographic separation was conducted on Shimadzu (Prominence LC 20 UFLC XR) connected with PDA detector; using column C18; Waters, (300 x 3.9 mm, 10 µm). The isocratic mobile phase consisted of ion pair reagent: Acetonitrile in ratio of (35: 65 v/v). Ion pair reagent is composed of 5.65 g of sodium hexane sulfonate and 2.75 g of sodium dihydrogen phosphate in 900 ml of water, adjusted to pH 2.3 with Phosphoric acid 85%, diluted to 1000 ml with water and filtered through 0.45 µm filter. The mobile phase was delivered to the system at a flow rate of 2 ml/min. An injection volume of 20 µl was used for Lumefantrine. The detection was carried out by PDA detector 342 nm. The calibration2 curve of Lumefantrine in mobile phase was linear with correlation coefficient (r) = 0.99946; over a concentration range of 60 – 1200 mg/l; with a retention time of 3.686 minutes. The percentage recovery of Lumefantrine was 100.029%. The relative standard deviation (RSD) was found to be less than 2. The proposed method was validated and successfully applied for determination of Lumefantrine in tablet dosage form.

4. **Mohamed Aly Amin Ahmed Ibrahim et al** [16] developed a HPLC Method D and did Validation for Determination of Artemether in Pharmaceutical Dosage Forms, it described a fast, precise and simple HPLC method was developed for estimation of Artemether in pure and pharmaceutical dosage forms. The chromatographic separation was conducted on Shimadzu (Prominence LC 20

HPLC) connected with Detector is ELSD with nitrogen pressure =360 Kpa; and temp = 37℃; Gain = 7; using column C18; Waters µ Bondapak, (300×3.9 mm, 10 µm). The isocratic mobile phase consisted of Acetonitrile: water in ratio of (90: 10, v/v). The mobile phase was delivered to the system at a flow rate of 1.5 ml/min. An injection volume of 20 µl was used for Artemether. Column Temperature was 37°C. The detection was carried out using ELSD detector. The calibration curve of Artemether in mobile phase was linear with correlation coefficient (r2) of 0.9987; over a concentration range of 1100–2000 mg/L; with a retention time of2.52 min. The percentage recovery of Artemether was 96.34%. The relative standard deviation (RSD) was found to be less than 2. The proposed method was validated and successfully applied for determination of Artemether in tablet dosage form.

5. **T.M. Kalyankar et al**[17] had done a simple, rapid, precise and accurate reversed phase high performance liquid chromatographic method has been developed for the simultaneous determination of Artemether in combination with Lumefantrine. This method uses a Hypersil ODS C18 (250mm ~4.6mm ~5µ particle Size) analytical column, a mobile phase of methanol: 0.05 % trifluroacetic acid with triethylamine buffer pH 2.8 adjusted with ortho phosphoric acid in ratio (80:20 v/v). The instrumental settings are a flow rate of 1.5 ml/min and PDA detector wavelength at 210 nm**.** The retention times for Artemether and Lumefantrine are 6.15 min and 11.31min, respectively. The method is validated and shown to be linear. The linearity range for Artemether and Lumefantrine are 20-120 & 120-720 µg/ml respectively. The Percentage recovery for Artemether and Lumefantrine are

ranged between 99.50–101.16 and99.78–101.21 respectively. The correlation coefficients of Artemether and Lumefantrine are 0.999, and 0.999,respectively**.** The relative standard deviation for six replicates is always less than 2%. The Statistical analysis proves that the method is suitable for analysis of Artemether and Lumefantrine as a bulk drug and in pharmaceutical formulation without any interference from the excipients.

6. **J Sunil et al**[18] had done a simple and precise HPLC method was developed for the estimation of Artemether and Lumefantrine in pure and pharmaceutical dosage forms. The quantification was carried out using symmetry C18, 250 x 4.6 mm, i.d, 5µm particle size in isocratic mode, with mobile phase compressing of buffer and Acetonitrile in the ratio of 40:60 (v/v), pH 3 ± 0.5. The flow rate was 1.5 ml/min and the detection was carried out by UV detector dual i.e, 210 and 303 nm. The retention times were 13.887 and 7.218 minutes for Artemether and Lumefantrine, respectively. The percentage recovery was found to be 98.87 and 99.78 % for Artemether and Lumefantrine; respectively. The method was validated by evaluation of different parameters.

7. **M Laxmi et al**[19] had done analytical method development and validation of Artemether in bulk drug, the method was found to be linear in the concentration range of 100-600 µg/ml, in the linearity study regression equation was found to be y = 0.199x – 1.133 & correlation coefficient was found to be 0.999. This method was Rugged and Robust in different testing criteria, LOD and LOQ were found to be 23.037µg/ml, 69.809µg/ml

respectively. Accuracy study was done in 3 different concentration level i.e 50, 100, 150% & % recovery of the method was found to be 99.4%, 100.4%, 99.7% respectively in 3 different levels & mean recovery was 99.8%, so method was accurate. A Simple and precise HPLC method was developed for the estimation of Artemether and Lumefantrine in pure and pharmaceutical dosage forms. The quantification was carried out using symmetry C18, 250 x 4.6 mm, i.d, 5µm particle size in isocratic mode, with mobile phase compressing of buffer and Acetonitrile in the ratio of 40:60 (v/v), pH 3 ± 0.5. The flow rate was 1.5 ml/min and the detection was carried out by UV detector dual i.e, 210 and 303 nm. The retention times were 13.887 and 7.218 minutes for Artemether and Lumefantrine, respectively. The percentage recovery was found to be 98.87 and 99.78 % for Artemether and Lumefantrine; respectively. The method was validated by evaluation of different parameters.

8. **R Arun et al**[20] had done development of analytical method for Lumefantrine by UV spectrophotometry, A Simple, sensitive, specific, spectrophotometric method has been developed for the detection of Lumefantrine in pure form and Pharmaceutical formulations. The optimum condition for the analysis of the drug was established. Lumefantrine exhibiting absorption at 234nm and obeyed beers law in the concentration range 8 to 16µg/ml. The lower limit of detection was found to be 4.3×10-2 and the limit of quantification to be 13.2 ×10-2. The regression equation was y = 0.065*x* + 0.02. The precision of the method was found to be 480.96mg at 234nm against the label claim of 480mg . The sample solution was stable up to 24 hours. The assay results were found

to be in good agreement with label claim. The proposed method was simple sensitive, precise, quick and useful for routine quality control.

9. **Isabella da Costa Ce´ sar et al**[21] did a simultaneous determination of Artemether and Lumefantrine in fixed dose combination tablets by HPLC with UV detection, this paper described the development and validation of a HPLC-UV method (210 nm) for the simultaneous quantitation of Artemether and Lumefantrine in fixed dose combination tablets. The method showed to be linear ($r2 > 0.99$), precise (R.S.D. < 2.0%), accurate (recovery of 101.07% for Artemether and 101.58%for Lumefantrine), specific and robust. Four batches of Artemether–Lumefantrine tablets were assayed by the validated method. The Artemether contents in the tablets varied from 98.61% to 103.35%, while Lumefantrine contents were 97.92–100.48%.

10. **B Nasir et al**[22] had done a new HPLC method for the determination of Artemether in injections, this new economical HPLC method has been developed for the estimation of Artemether in injections to reduce the cost of estimation. Previously, one HPLC method is available in European Pharmacopoeia for the estimation of Artemether but it is costly due to high price of Acetonitrile. Present study replaced Acetonitrile with methanol and showed that new method remained as specific, linear, accurate and precise as previous.

11. **Naveen S Kotur et al**[23] had done Analytical Method Development and Validation for Estimation of Lumefantrine in Pharmaceutical Dosage Forms

by HPLC A Simple, rapid, sensitive, precise, accurate, stability indicating and reproducible of High Performance Liquid chromatography(HPLC) method has been developed for the estimation of Lumefantrine in pharmaceutical Tablet dosage forms. The HPLC method was carried out using Waters Symmetry C18 (250 X 4.5 mm) analytical column with maintained the column oven temperature 35 °C and isocratic pump mode. The mobile phase compressing of water, acetonitrile and glacial acetic acid in the ratio of 48: 52: 1, v/v/v with delivered the flow rate of 1.2mL /min and the detected the Lumefantrine at 276 nm from PDA detector. The retention time of Lumefantrine was 4.65 minutes. This method has been validated as per ICH guidelines and the validation data showed that the assay is sensitive, specific and reproducible for the determination of Lumefantrine in the dosage form. The method is linear from 10µgmL-1 to100µgmL-1 and linear correlation coefficient (R2) was more than 0.9990. The accuracy of the method by recovery was found between99.44 and 100.14 %. Mean inter and intraday assay relative standard deviation (RSD) were less than 1.0%. The proposed method provided an accurate and precise analysis of Lumefantrine in its Pharmaceutical dosage form.

12. **Gupta N.K et al**[24] had done simultaneous determination of Artemether and Lumefantrine by RP-HPLC method development in pharmaceutical tablet dosage form, the chromatographic analysis was performed by Hypersil BDS C18, 250 × 4.6 mm, 5 µ particle size with mobile phase consisting of buffer and Acetonitrile in the ratio of 50:50v/v, orthophosphoric acid used as buffer (pH 3.0 + 0.6), at a flow rate of 1.5 ml/min and eluents monitored at 215nm.

The method was validated for linearity, accuracy, precision, robustness and application for assay as per ICH guidelines. The retention times of Artemether and Lumefantrine were 2.464 and 6.236 min, respectively. The calibration curves of peak area versus concentration, which was linear from 4-24µg/ml for Artemether and 24-144µg/ml for Lumefantrine, had regression coefficient (r2) greater than 0.999. The method had the requisite accuracy, precision, and robustness for simultaneous determination of Artemether and Lumefantrine in tablets. The proposed method is simple, economical, accurate and precise, and could be successfully employed in routine quality control for the simultaneous analysis of Artemether and Lumefantrine in tablets.

13. **B Sridhar et al[25]** had done a validated reverse phase HPLC method for the simultaneous estimation of Artemether and Lumefantrine in pharmaceutical dosage forms, a simple, sensitive and precise reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Artemether and Lumefantrine in pharmaceutical dosage forms. The mobile phase consisted of Acetonitrile: buffer (0.1% v/v ortho phosphoric acid, PH – 3) in the ratio of 60:40 v/v delivered at a flow rate of 1.5 ml / min and wavelength of detection at 303 nm. The retention times of Artemether and Lumefantrine were 13.888 min and 7.207 min respectively. The developed method was validated according to ICH guidelines. The proposed method can be used for determination of these drugs in combined dosage forms.

14. **P Umapathi et al[26]** had done development and Validation of a Dissolution Test Method for Artemether and Lumefantrine in Tablets, a single dissolution

method for evaluating the in vitro release of Artemether and Lumefantrine from tablets was developed and validated. The method comprised of a dissolution medium of 1000 ml of2 %w/v of Myrj 52 in 0.005M HCl per vessel with the paddle rotating at 100 rpm for 120 min. The dissolution samples were analysed using a Waters HPLC system with Waters symmetry column (C-18column of 250mm x 4.6mm i.d., 5 µ particle size). The mobile phase was a mixture of 20 volumes of 0.5%v/v of tri ethylamine in water (adjusted to a pH of 3.0 with ortho phosphoric acid) and 80 volumes of Acetonitrile. The detection wavelength was set at 216 nm and 100 µl of each sample was injected. The HPLC method used for the determination of drug release was validated for the parameters of accuracy, precision, linearity, specificity, filter validation, solution stability and robustness.

15. **Isabella da Costa César et al**[27] had done Robustness evaluation of the chromatographic method for the quantitation of Lumefantrine using Youden's test, it is a reliable method to evaluate the robustness of analytical methods, by means of an experiment design which involves seven analytical parameters combined in eight tests. In the present study, we assessed the robustness of a chromatographic method to quantify Lumefantrine in raw material samples, using Youden's test. Hence, it was possible to determine the effect of each analytical parameter in the final analysis results. Youden's test showed to be a simple and feasible procedure to evaluate the robustness of chromatographic methods.

16. **MannurVinodh et al**[28] had done Analytical Method Development And Validation For Simultaneous Estimation Of Artemether And Lumefantrine In Pure And Pharmaceutical Dosage Form Using RP-HPLC Method, A simple, rapid, precise and cost effective reversed phase-high performance liquid chromatography (RP-HPLC) method was developed for the simultaneous estimation of Artemether (AT) and Lumefantrine (LU) in pure drug and pharmaceutical dosage forms. The separation was carried out using BDS Hypersil C18 (150 × 4.6 mm i.d. 3 µm particle size) column, with mobile phase comprising of 0.01M tetra butyl ammonium hydrogen sulphate and acetonitrile in the ratio of 20 : 80 (v/v). The flow rate was 1.0ml/min and the detection was carried out using UV-visible detector at 222 nm. The method was validated by evaluation of different parameters such as accuracy, precision, linearity, ruggedness, and robustness, limit of detection (LOD) and limit of quantification (LOQ). The retention time were found to be 4.19 and 5.22 min for AT and LU, respectively. Correlation coefficient (r2) of 0.999 for both over concentration range of 3.2-19.2µg/ml and 16-96µg/ml for AT and LU, respectively. Parameters like mobile phase ratio, wavelength, flow rate, etc. were deliberately varied. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed RP-HPLC method is robust. Intra and inter day precision reproducibility study was carried out and it was checked by determining precision on the same instrument, but by a different analyst. The percentage recovery for AT and LU were ranged between 99.18-100.19 and 99.96-100.07, respectively. The LOD for AT and LU were found to be 0.201 and 2.99 µg/ml and the LOQ were 0.609 and 9.086 µg/ml respectively. Method was found to be reproducible

with relative standard deviation (RSD) for intra and inter day precision less than 2%.

17. **Mathieu Verbeken et al**[29] Stability-indicating HPLC-DAD/UV-ESI/MS impurity profiling of the anti-malarial drug Lumefantrine, it is a fluorine derivative belonging to the aryl amino alcohol class of anti-malarial drugs and is commercially available in fixed combination products with β-Artemether. Impurity characterization of such drugs, which are widely consumed in tropical countries for malaria control programes, is of paramount importance. However, until now, no exhaustive impurity profile of Lumefantrine has been established, encompassing process-related and degradation impurities in active pharmaceutical ingredients (APIs) and finished pharmaceutical products (FPPs).

18. **Karen Gaudin et al**[30] had done Simultaneous Determination of Artemether and Azithromycin in Suppositories by Reversed Phase HPLC, Chromatographic parameter assessments for RP-HPLC-UV method development for the simultaneous analysis of Artemether and Azithromycin for the pharmaceutical analysis of a rectal co formulation currently under development for the treatment of malaria infected children. Using methanol based mobile phase for the analysis of both Artemether and Azithromycin provided a more robust method in terms of resolution and peak symmetry. The method validated for suppository used 80% methanol and 20% phosphate buffer 15 mM at pH 9. The UV detection was at 210 nm. The accuracy profiles indicated a method validation between 80–120% for both active

pharmaceutical ingredients. The preparation process of the suppository was validated based on theoretical values of Artemether and Azithromycin present in the formulation; active pharmaceutical ingredients were homogenously distributed within the suppository.

3. AIM AND PLAN OF WORK

The aim of the work is to develop a new method for the analysis of Artemether and Lumefantrine by RP–HPLC in powder for oral suspension dosage form (dry syrup) and to validate it as per ICH guidelines**.**

Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw materials used and the final product obtained meets their appropriate specification. The number of drugs and drug formulations introduced into the market has been increased and that product has been analyzed as per validated method. These drugs or formulations may be either new entities in the market or partial structural modification of the existing drugs or novel dosage forms or multi component dosage forms.

The multi component dosage forms proved to be effective due to combined mode of action on the body. The complicity of including the presence of multiple drug entities posses considerable challenge to the analytical chemist during the development of assay procedure. The estimation of individual drugs in these multi component dosage forms becomes difficult due to some extraction or isolation procedures.

For the present study Artemether and Lumefantrine was selected. The extensive literature survey carried out revealed that there is no method reported for the simultaneous estimation of these drugs in powder for oral suspension, some methods for estimation of combined drugs by HPLC and spectrophotometer are available. Hence present study aim to developing a specific, precise, accuracy, linear,

simple, rapid, and validated and cost effective RP-HPLC method for the simultaneous estimation of these drugs in combined dosage forms.

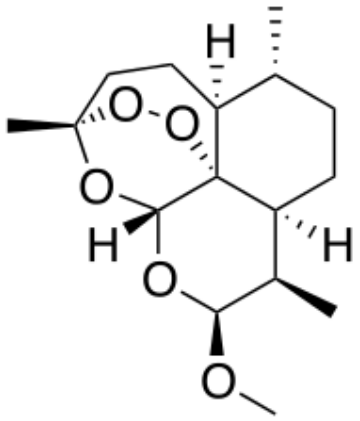
The core of project for RP-HPLC method was designed as follows:

1. Selection of suitable wavelength,
2. Selection of mobile phase,
3. Selection of initial separation conditions,
4. Optimization of chromatographic conditions,
5. Estimation of Artemether and Lumefantrine
6. Method validation
    - Specificity
    - Linearity and Range
    - Precision
    - Accuracy
    - Ruggedness
    - Limit of detection
    - Limit of quantitation
    - Robustness

4. DRUG PROFILE

ARTEMETHER

Structure :



Molecular Formula : $C_{16}H_{26}O_5$

IUPAC name : (3*R*,5a*S*,6*R*,8a*S*,9*R*,10*S*,12*R*,12a*R*)-10-methoxy-3,6,9-trimethyldecahydro-12*H*-3,12-epoxy[1,2] dioxepino [4,3-*i*]-2-benzopyran.

Molecular weight : 298.374 g/mol

Description : White crystalline powder..

Solubility : Insoluble in Water

pKa(Strongest basic) : 3.9

Category : Anti malarial.

Pharamacokinetic data:

- Absorption**:** Absorption of Artemether is improved 2- to 3-fold with food. It is highly bound to protein (95.4%). Peak concentrations of Artemether are seen 2 hours after administration
- Metabolism**:** Artemether is metabolized in the human body to the active metabolite, dihydroartemisinin, primarily by hepatic enzymes CYP3A4/5.Both the parent drug and active metabolite are eliminated with a half-life of about 2 hours
- Elimination**:** Artemether cleared from plasma with an elimination half-life of about 2 hours.ntrine.

Mechanism of action:

Antimalarial agent Artemether is rapidly metabolized into an active metabolite dihydroartemisinin (DHA). The antimalarial activity of Artemether and DHA has been attributed to endoperoxide moiety.

Toxicity:

Impairment of Fertility: Pregnancy rates were reduced by about one-half in female rats dosed for 2 to 4 weeks with the Artemether Lumefantrine combination at 1000 mg/kg (about 9 times the clinical dose based on body surface area comparisons). Male rats dosed for 70 days showed increases in abnormal sperm (87% abnormal) and increased testes weights at 30 mg/kg doses (about one-third the clinical dose). Higher doses (about 9 times the clinical dose) resulted in decreased sperm motility and 100% abnormal sperm cells.

Uses:

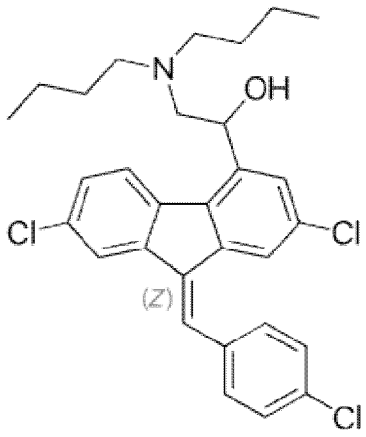
- Artemether is an antimalarial drug for uncomplicated malaria caused by P. falciparum (and chloroquine-resistant P. falciparum) or chloroquine-resistant P. vivax parasites. Artemether can also be used to treat severe malaria.
- The World Health Organization recommends the treatment of uncomplicated P. falciparum with artemisinin-based combination therapy.[11] Given in combination with lumefantrine, it may be followed by a 14 day regimen of primaquine to prevent relapse of *P. vivax* or *P. ovale* malarial parasites and provide a complete cure.
- Artemether can also be used in treating and preventing trematode infections of schistosomiasis when used in combination with praziquantel.

Storage:

Store in a cool, dry place. Store in a tightly closed container. Recommended storage temperature: -20°C

LUMEFANTRINE

Structure



Molecular Formula : $C_{30}H_{32}Cl_3NO$

IUPAC Name **:** 2-(Dibutylamino)-1-[(9Z)-2,7-dichloro-9-(4-chlorobenzylidene)-9H-fluoren-4-yl]ethanaol.

Molecular weight **:** 528.939 g/mol

Description **:** Pale pink colored amorphous solid

Solubility **:** Soluble in Dimethyl sulfoxide

pKa (strongest acidic): 14.1

pKa (strongest basic) : 9.78

Category **:**Anti malarial

Mechanism of action:

Lumefantrine is a blood schizonticide active against erythrocytic stages of *Plasmodium falciparum.* It is thought that administration of Lumefantrine with Artemether results in cooperate antimalarial clearing effects. Artemether has a rapid onset of action and is rapidly cleared from the body. It

is thus thought to provide rapid symptomatic relief by reducing the number of malarial parasites. Lumefantrine has a much longer half life and is believed to clear residual parasites.

Pharamacokineticdata :

Absorption: Absorption of lumefantrine, a highly lipophilic compound, starts after a lag-time of up to 2 hours, with peak plasma concentrations about 6 to 8 hours after administration.

Metabolism: Lumefantrine was metabolized mainly by CYP3A4 to desbutyl Lumefantrine. The systemic exposure to the metabolite desbutyl-Lumefantrine was less than 1% of the exposure to the parent compound.

Elimination: Lumefantrine is eliminated more slowly, with an elimination half-life of 3 to 6 days.

Uses: It is used to treat**malaria** caused by Plasmodium falciparum

Storage: Store in well-closed light-resistant containers at -20 ^{0}C

5. MATERIALS AND INSTRUMENT USED

5.1 CHEMICAL AND SOLVENTS USED:

Table 5.1

S. No.	Name	Grade	Make	Lot Number	Purity
01	1-Hexane sulphonic acid sodium salt anhydrous	HPLC	Finar	96170713a15	99.00%
02	Monobasic sodium phosphate anhydrous	AR	Himedia	0000194436	98.00%
03	Acetonitrile	HPLC	Finar	1285161012A0	99.80%
04	Orthophosphoric acid	AR	Rankem	G125D15	88.00%
05	Ethanol	AR	Jiangsu Huaxi International	20141205	99.90%
06	HPLC water	AN water			

5.2 COLUMN USED:

Table 5.2

S.No.	Make	Column Name	ID	Serial No.
01	Agilent	Zorbax SB-C8, 5µm, 250mm x 4	AD/LC CN/057	USSH016106

5.3 SAMPLE USED:

Table 5.3

Product name	Batch number	Mfg. Date	Exp. Date
Artemether and Lumefantrine for oral suspension(180 mg & 1080 mg)	FDPS590315	Mar-2015	NA

5.4 WORKING STANDARD, PLACEBO USED:

Table 5.4

.No.	Name	WS code	Purity
01	Artemether Working Standard	CP2WS/080/00	99.41%
02	Lumefantrine Working Standard	CP2WS/078/00	99.47%
03	Placebo	Not Applicable	

5.5 INSTRUMENTS USED:

Table 5.5

S.No.	Name	Make & Model	ID
01	Analytical Weighing Balance	Mettler Toledo XS105	AD/INS/001
02	HPLC	Agilent 1260 & 1290 series	AD/INS/030
03	pH Meter	Hanna	AD/INS/035
04	Sonicator	PCI India	AD/INS/016

6. METHOD DEVELOPMENT AND VALIDATION

Solubility

Artemether is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide, which should be purged with an inert gas. The solubility of Artemether in these solvents is approximately 10 and 20 mg/ml, respectively. Artemether is sparingly soluble in aqueous buffers. According to literature review Lumefantrine very slightly soluble in acetonitrile and soluble in chloroform and dichloromethane, and it is practically insoluble (0.002%) in water. Finally buffer pH2.3, Ethanol and Acetonitrile in the ratio of 100:100:300 was selected as a solvent.

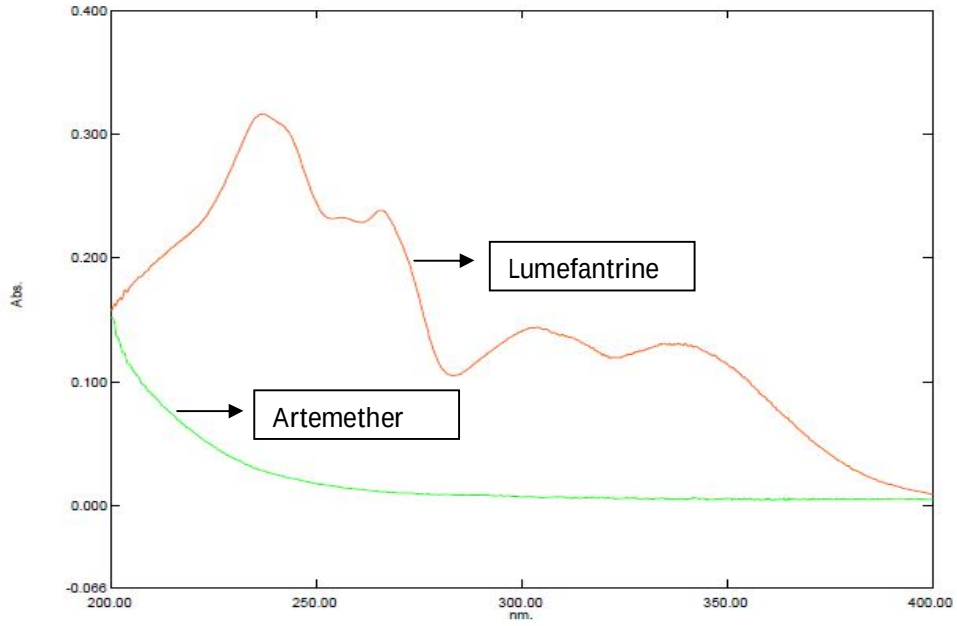
Selection of Mobile Phase

The pure drugs Artemether and Lumefantrine were injected in combination in the ratio of their contents in the formulation to the chromatographic system, and run in different mobile phase compositions. Different mobile phases containing different compositions of methanol: water, acetonitrile: water, acetonitrile: Phosphate buffer 6.8 and acetonitrile: Phosphate buffer pH 2.3 was tried for the selection of optimum conditions for the simultaneous determination of Artemether and Lumefantrine. It was found that optimal separation of the two components was achieved with acetonitrile and phosphate buffer, compared to other mobile phases. Different ratios of the selected mobile phase were tried with varying flow rates and pH. Finally, the mobile phase composition selected for the chromatographic separation of Artemether and Lumefantrine was acetonitrile and phosphate buffer of pH 2.3 in the ration of 45:55 v/v.

Selection of Analytical Wavelength

UV Spectrophotometric determination of Artemether and Lumefantrine individually, shows that both the drugs absorb appreciably at 210nm, hence 210nm was selected as the detection wavelength.

Figure: 6.1 UV Spectra of Artemether and Lumefantrine



Optimized Chromatographic Conditions

Chromatographic conditions:

Column : C_8, 15 cm x 4.6-mm, 5-µm [Zorbax SB-C_8 is suitable]

Flow Rate : 2.0 mL/minute

Pump mode : Isocratic

Detector Wavelength : 210 nm

Injection volume : 20 µL

Column Temperature : 40°C

Sample Temperature : 20°C

Mobile phase : Prepared a mixture 450 volume of buffer (pH:2.3) and 550 volume of Acetonitrile, mixed. Filtered the solution through 0.45 µm nylon filter and sonication was done for 10 minutes.

Methodology adopted

- Preparation of buffer solution
- Preparation of mobile phase
- Preparation of diluent
- Preparation of standard stock solution
- Preparation of standard solution
- Preparation of sample solution
- Preparation of Placebo solution
- Setting the instrumental parameters before performing the analysis for Detector and Pump
- Development of chromatogram and determination of retention time.

Preparation of buffer (pH 2.3):

Weighed and dissolved 5.65 g of 1-Hexane sulphonic acid sodium salt anhydrous and 2.75 gm of sodium phosphate monobasic anhydrous in 1000 mL of purified water, mixed well. Adjusted the pH to 2.3 with dilute phosphoric acid.

Preparation of dilute Orthophosphoric acid:

Transferred 6.9 mL of orthophosphoric acid through pipette into 100 mL volumetric flask and diluted up to the volume with water.

Preparation of Mobile phase:

Prepared a mixture 450 volume of buffer and 550 volume of Acetonitrile, mixed. Filtered the solution through 0.45 µm nylon filter and sonication was done for 10 minutes.

Preparation of Diluent:

Prepared a mixture of buffer, Ethanol and Acetonitrile in the ratio of 100:100:300. Filtered the solution through 0.45 µm nylon filter and sonication was done for 10 minutes and mixed.

Procedure for preparation of analytical solutions

Preparation of standard solution 320.0 mcg/mL of Artemether and 120.0 mcg/mL of Lumefantrine

Weighed accurately 80 mg of Artemether working standard, 30 mg of Lumefantrine working standard and transferred into 250 mL volumetric flask, added 100 mL of diluent and sonication was done for 15 minutes and dissolved, cooled and diluted up to the volume with diluent and mixed. Filtered the solution through 0.45 µm nylon filter and collected the solution in HPLC vial after discarded first 2 mL of the filtrate.

Estimation of sample solution of Artemether 320.0 mcg/mL and Lumefantrine 120.0 mcg/mL

Constituted Artemether and Lumefantrine for Oral Suspension with water up to the volume specified in the labeling. Weighed accurately about 80 mg (about 29.46 g of sample) for Artemether and 30 mg (about 1.85 g of sample) for Lumefantrine transferred in to 250 mL volumetric flask respectively. Added 120 mL of diluent to

each and shaked by mechanical for 30 minutes and sonication was done for 20 minutes with intermediate shaking and dissolved, cooled and diluted up to volume with diluent. Mixed and filtered the solution through 0.45 µm Nylon filter and collected the sample solution in HPLC vial after discarded first 2 mL of the filtrate. The chromatogram was showed in chromatogram no.7.7 and 7.8.

Placebo solution for Artemether and Lumefantrine

Weighed accurately 29.8267 g of placebo for Artemether and 1.8391 g of placebo for Lumefantrine (based on density 1.1215g/mL) and transferred into 250 mL volumetric flask respectively. Added 120 mL of diluent to each and shaked by mechanical for 30 minutes and sonication was done for 20 minutes with intermediate shaking and dissolved, cooled and diluted up to volume with diluent. Mixed and filtered the solution through 0.45 µm Nylon filter and collected the sample solution in HPLC vial after discarded first 2 mL of the filtrate. The chromatogram was showed in chromatogram no.7.5.

CALCULATIONS

Content of Artemether in mg:

SPL Area STD wt in mg 250 STD Purity in ASB

= ---------- X---------- X ---------- X ---------- x Weight per mL x 60

STD Area 250 SPL wt in g 100

Content of Artemether in Percentage (%):

$$= \frac{\text{Artemether in mg}}{\text{Label claim of Artemether in each 60 mL contains in mg.}} \times 100$$

Content of Lumefantrine in mg:

$$= \frac{\text{SPL Area}}{\text{STD Area}} \times \frac{\text{STD wt in mg}}{250} \times \frac{\text{STD Purity in ASB}}{\text{SPL wt in g}} \times \text{Weight per mL} \times 60$$

Content of Lumefantrine in Percentage (%):

$$= \frac{\text{Lumefantrine in mg}}{\text{Label claim of Lumefantrine in each 60 mL contains in mg}} \times 100$$

METHOD VALIDATION

Validation of analytical method is a process to establish that the performance characteristics of the developed method meet the requirement of the intended analytical application. (Lloyd R. Synder, 1997)[4] (ICH Harmonized Tripartite Guidelines, 2005)[6]

SYSTEM SUITABILITY PARAMETERS

System suitability is the test to ensure that the methods can generate results of acceptable accuracy and precision. (Lloyd R. Synder, 1997) [4]

Parameters studied

Tailing Factor.

Number of theoretical plates (N).

Retention time.

Relative standard deviation.

Typical analytical parameters used in method validation include

1. Specificity
2. Linearity and Range
3. Precision
4. Accuracy
5. Ruggedness
6. Limit of detection
7. Limit of quantitation
8. Robustness

SPECIFICITY

The specificity of the method can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample materials. (Lloyd R. Synder, 1997)[4] (ICH Harmonized Tripartite Guidelines, 2005)[6]

Procedure:

Inject the blank, placebo, standard and sample preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Table No: 6.1 Injection sequence for Specificity

Particulars	Number of Injection
Blank	1
Placebo preparation	1
Standard preparation	5
Sample preparation	2
Bracketing standard	1

Acceptance criteria:

No any peak should be obtained in the retention time of Artemether and Lumefantrine from the blank and placebo chromatograms.

LINEARITY AND RANGE

Linearity is the measure of how well a calibration plot of response Vs concentration approximates a straight line. Linearity can be assessed by performing the single measurement s at several analyte concentrations. The data are then processed using a linear least square regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

Ability to obtain test results which are directly proportional to the concentration of analyte. For an establishment of the linearity 60%, 80%, 100%, 120%, and 160% of standard and sample concentrations shall be used.

Preparation of Linearity from standard:

Standard stock solution

Weighed accurately 160 mg of Artemether working standard, 60mg of Lumefantrine working standard and transferred into 250 mL volumetric flask, added 100 mL of diluent and sonication was done for 15 minutes and dissolved, cooled and diluted up to the volume with diluent and mixed.

60 % Linearity standard concentration (192.0 mcg/mL of Artemether and 72.0 mcg/mL of Lumefantrine):

Transfer 3.0 mL of above standard stock solution through pipette into a 10 mL volumetric flask and dilute up to the volume with diluent and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

80 % Linearity standard concentration (256.0 mcg/mL of Artemether and 96.0 mcg/mL of Lumefantrine):

Transfer 4.0 mL of above standard stock solution through pipette into a 10 mL volumetric flask and dilute up to the volume with diluent and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about first 2 mL of filtrate.

100 % Linearity standard concentration (320.0 mcg/mL of Artemether and 120.0 mcg/mL of Lumefantrine):

Transfer 5.00 mL of above standard stock solution through pipette into a 10 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the

solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

120 % Linearity standard concentration (384.0 mcg/mL of Artemether and 144.0 mcg/mL of Lumefantrine):

Transfer 6.0 mL of above standard stock solution through pipette into a 10 mL volumetric flask and dilute up to the volume with diluent and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

160 % Linearity standard concentration (512.0 mcg/mL of Artemether and 192.0 mcg/mL of Lumefantrine):

Transfer 8 mL of above standard stock solution through pipette into a 10 mL volumetric flask and dilute up to the volume with diluent and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

Acceptance criteria:

- Correlation co-efficient between concentrations and its area should be more than 0.998.

PRECISION

Precision is the measure of the repeatability of result. The precision of an analytical procedure expresses the closeness of agreement between a series of measurements under the prescribed conditions.

SYSTEM PRECISION

Determines the closeness of agreement of the same homogenous standard preparations under the prescribe conditions.

Procedure:

Inject the blank, standard preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Table No: 6.2 Injection sequence for System Precision

Particulars	Number of Injection
Blank	1
Standard preparation	6

Acceptance criteria

- The Tailing factor of the peak due to Artemether and Lumefantrine obtained from five replicates standard solution injections should be not more than 3.0.
- Theoretical plates of the peak obtained for five replicates standard solution injections of Artemether and Lumefantrine should be not less than 2000.
- The relative standard deviation of the area obtained for Five replicate standard solution of Artemether and Lumefantrine should be not more than 2.0%.
- The relative standard deviation of the retention time obtained for five replicate standard solution of Artemether and Lumefantrine should be not more than 1.0%.

METHOD PRECISION (REPEATABILITY)

Determines the closeness of agreement of the same homogenous sample under the prescribe conditions. Performing assay of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg), a minimum of six sample preparation from a single batch shall be made and analyze separately.

Procedure

Inject the blank, standard and sample preparations based on "Injection sequence" detailed below and measure the corresponding area.

Table No: 6.3 Injection sequence for Method Precision

Particulars	Number of Injection
Blank	1
Standard Preparation	6
Sample preparations for Artemether– 1, 2, 3, 4, 5 & 6	Each 2
Sample preparations for Lumefantrine– 1, 2, 3, 4, 5 & 6	Each 2
Bracketing Standard	1

Acceptance criteria:

- Assay obtained for each six sample preparations should be between 90.0 and 110%
- The RSD obtained for the assay results for six sample preparations should be not more than 2.0 %.

ACCURACY

Accuracy is defined as closeness of measured value to the true value. The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Preparation of analytical solution

Preparation of Standard solution (320.0 mcg/mL of Artemether and 120.0 mcg/mL of Lumefantrine)

Weigh accurately about 80.0 mg of Artemether working standard, 30 mg of Lumefantrine working standard, and transfer in to 250 mL volumetric flask, Add 100 mL of diluent and sonicate for 15 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well. Filter the solution through 0.45 µm Nylon filter and collect the sample solution in HPLC vial after discarding about first 2 mL of the filtrate.

Accuracy Sample preparation for Artemether

Placebo spiked with 50 % standard solution preparation (160.0 mcg/mL of Artemether)

Accurately weigh 40 mg of Artemether WS, 29.8267g of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg) placebo (based on density 1.1215g/mL) and transfer into 250 mL volumetric flask. Add about 100 mL of diluent and sonicate for 20 minutes with intermediate shaking to dissolve. Cool and dilute up to volume with diluent. Filter the solution through 0.45 µm Nylon filter and collect

the sample solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 100 % standard solution preparation (320.0 mcg/mL of Artemether)

Accurately weigh 80 mg of Artemether WS, 29.8267g of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg) placebo (based on density 1.1215g/mL) and transfer into 250 mL volumetric flask. Add about 100 mL of diluent and sonicate for 20 minutes with intermediate shaking to dissolve. Cool and dilute up to volume with diluent. Filter the solution through 0.45 µm Nylon filter and collect the sample solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 150 % standard solution preparation (480.0 mcg/mL of Artemether)

Accurately weigh 120 mg of Artemether WS, 29.8267g of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg) placebo (based on density 1.1215g/mL) and transfer into 250 mL volumetric flask. Add about 100 mL of diluent and sonicate for 20 minutes with intermediate shaking to dissolve. Cool and dilute up to volume with diluent. Filter the solution through 0.45 µm Nylon filter and collect the sample solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Accuracy Sample preparation for Lumefantrine

Placebo spiked with 50 % standard solution preparation (60.0 mcg/mL of Lumefantrine)

Accurately weigh 15 mg of Lumefantrine WS, 1.8391g of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg) placebo (based on density 1.1215g/mL) and transfer into 250 mL volumetric flask. Add about 100 mL of diluent and sonicate for 20 minutes with intermediate shaking to dissolve. Cool and dilute up to volume with diluent. Filter the solution through 0.45 µm Nylon filter and collect the sample solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 100 % standard solution preparation (120.0 mcg/mL of Lumefantrine)

Accurately weigh 30 mg of Lumefantrine WS, 1.8391g of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg) placebo (based on density 1.1215g/mL) and transfer into 250 mL volumetric flask. Add about 100 mL of diluent and sonicate for 20 minutes with intermediate shaking to dissolve. Cool and dilute up to volume with diluent. Filter the solution through 0.45 µm Nylon filter and collect the sample solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 150 % standard solution preparation (180.0 mcg/mL of Lumefantrine)

Accurately weigh 45 mg of Lumefantrine WS, 1.8391g of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg) placebo (based on density 1.1215g/mL) and transfer into 250 mL volumetric flask. Add about 100 mL of diluent

and sonicate for 20 minutes with intermediate shaking to dissolve. Cool and dilute up to volume with diluent. Filter the solution through 0.45 µm Nylon filter and collect the sample solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Procedure

Inject the blank, standard and placebo spiked standard preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Table: 6.4 Injection sequence for Accuracy

Particulars	**No. of injections**
Blank	1
Standard preparation	6
Placebo spiked with 50 % Artemether standard preparation 1, 2 & 3	Each 3
Placebo spiked with 100 % Artemether standard preparation 1, 2 & 3	Each 3
Placebo spiked with 150 % Artemether standard preparation 1, 2 & 3	Each 3
Placebo spiked with 50 % Lumefantrine standard preparation 1, 2 & 3	Each 3
Placebo spiked with 100 % Lumefantrine standard preparation 1, 2 & 3	Each 3
Placebo spiked with 150 % Lumefantrine standard preparation 1, 2 & 3	Each 3
Bracketing Standard	1

Calculations for Artemether accuracy:

Step 1: Standard added in mg (Working standard solution)

$$= \frac{\text{Standard wt in mg}}{250}$$

Step 2: Placebo spiked standard added in mg at 100%

$$= \frac{\text{Wt. of spiked standard in mg}}{250}$$

Step 3: Placebo spiked standard recovered in mg

$$= \frac{\text{Placebo spiked standard area X standard added in mg (Working standard solution)}}{\text{Average area of working standard solution}}$$

Step 4: Recovery in percentage

$$= \frac{\text{Placebo spiked standard recovered in mg}}{\text{Placebo spiked standard added in mg}} \times 100$$

Calculations for Lumefantrine accuracy

Step 1: Standard added in mg (Working standard solution)

$$= \frac{\text{Standard wt in mg}}{250}$$

Step 2: Placebo spiked standard added in mg at 100%

$$= \frac{\text{Wt. of spiked standard in mg}}{250}$$

Step 3: Placebo spiked standard recovered in mg

$$= \frac{\text{Placebo spiked standard area X standard added in mg (Working standard solution)}}{\text{Average area of working standard solution}}$$

Step 4: Recovery in percentage

$$= \frac{\text{Placebo spiked standard recovered in mg}}{\text{Placebo spiked standard added in mg}} \times 100$$

Acceptance criteria

In each concentration, the ranitidine hcl working standard spiked with placebo should be recovered between 98.0 % and 102.0 %.

RUGGEDNESS

Defined by USP, The Ruggedness is the degree of reproducibility of test results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory and from analyst to analyst.

Procedure

Working standard solutions and working sample solution were prepared by different analyst on different days. Solutions were injected as per the test method and chromatograms were recorded.

Inject the blank, standard and sample solution preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Table No: 6.5 Injection sequence for Ruggedness

Particulars	Number of Injection
Blank	1
Standard preparation	5
Sample preparations – 1, 2, 3 (Analyst 1 and 2)	Each 2
Bracketing Standard	1

Acceptance criteria:

- Assay in % obtained for each six sample preparations should be between 90.0 and 110 %
- The RSD obtained for the assay results for six sample preparations should be not more than 2.0 %.

ROBUSTNESS

Robustness of an analytical method is measure of its capacity to remain unaffected by small but deliberate variation in method parameters. It provides information about the reliability of method.

Determination

The robustness of an analytical method is determined by analysis of aliquots of homogenous lots by differing physical parameters like flow rate and column temperature.

Change in flow rate plus (2.2 mL/minute):

For Chromatographic conditions, follow the method of analysis except by changing the flow to 2.2 mL / minute instead of 2.0 mL / minute.

Change in flow rate minus (1.8 mL/minute):

For Chromatographic conditions, follow the method of analysis except by changing the flow to 1.8 mL / minute instead of 2.0 mL / minute.

Change in wavelength plus (212 nm):

For Chromatographic conditions, follow the method of analysis except by changing the wavelength to 212 nm instead of 210 nm.

Change in wavelength minus (208nm):

For Chromatographic conditions, follow the method of analysis except by changing the wavelength to 208nm instead of 210 nm.

Change in mobile phase organic content plus (+5%):

Change the least organic solvents ratio to 57.5 volumes instead of 55 volume, and adjust the quantity variation from Buffer concentration to maintain 100 %.

Change in mobile phase organic content minus (-5%):

Change the least organic solvents ratio to 52.25 volumes instead of 55 volumes, and adjust the quantity variation from Buffer concentration to maintain 100%.

Inject the blank, standard and sample solution preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Table No: 6.6 Injection sequence for Robustness

Particulars	**Number of Injection**
Blank	1
Standard solution	5
Sample solution	2
Bracketing standard solution	1

Acceptance criteria:

- Assay obtained for each robustness parameter should be between 90.0 and 110.0%.The combined RSD obtained for the assay result of an each robustness parameter and six assay results of method precision should be not more than 2.0 %.

STABILITY STUDIES

Stability of the sample and standard solutions used in HPLC method is required to generate reproducible and reliable results. The sample and standard solutions

were subjected to short term stability studies at room temperature. Stability studies were carried out initially, at 8 hours and 16 hours, 24 hours time lapse of solution preparation. This study should be performed by injecting standard solution and sample solution at probable time points, and minimum not less than 24 hours shall be studied.

Note:

Maintain the chromatographic conditions and solutions preparation as per the procedure given in method of analysis and injections sequence for solution stability study is given in below table.

The maximum time for inject able usage of standard and sample solution is absolutely depends on the **24 Hours** of solutions stability studied.

Injection sequence:

Table No: 6.7 Injection sequence for Solution Stability

Particulars	Number of Injections
Blank (0 Hour)	1
Standard preparation (0 Hour)	1
Sample preparation (0 Hour)	1
Blank (X Hours)	1
Standard preparation (X Hours)	1
Sample preparation (X Hours)	1

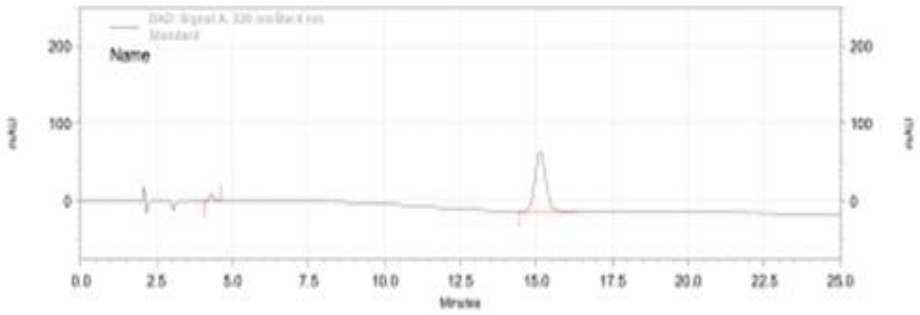
Acceptance criteria:

Cumulative % RSD for area obtained between initial time point and various probable intervals time points should be not more than 2.0 %.

7. CHROMATOGRAMS

Method Development

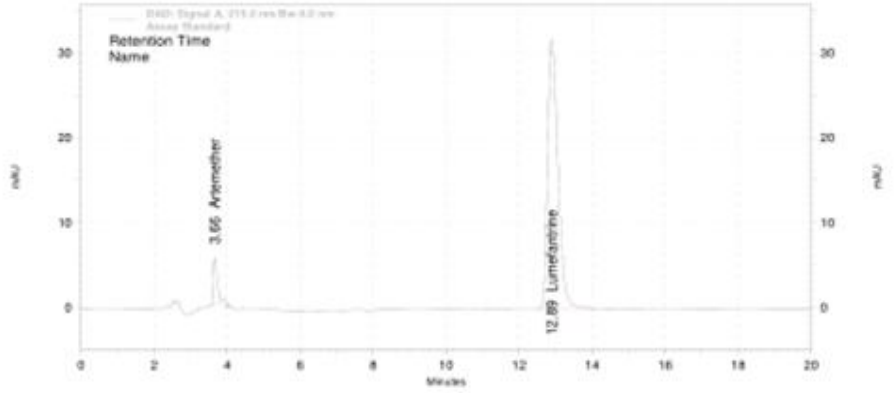
Chromatogram No – 7.1
Trail No.1



Results

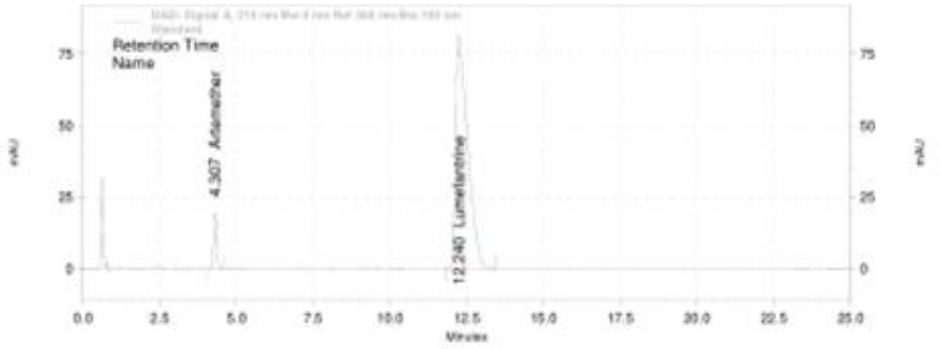
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	**Resolution (USP)**
1	Artemether	4.307	175353	5480	0.96	**0.00**
2	Lumefantrine	15.127	4026102	8637	1.13	**24.49**

Chromatogram No – 7.2
Trail No.2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	3.660	5429938	5213	1.67	0.00
2	Lumefantrine	12.893	83925461	9834	1.45	25.55

Chromatogram No – 7.3
Trail No.3



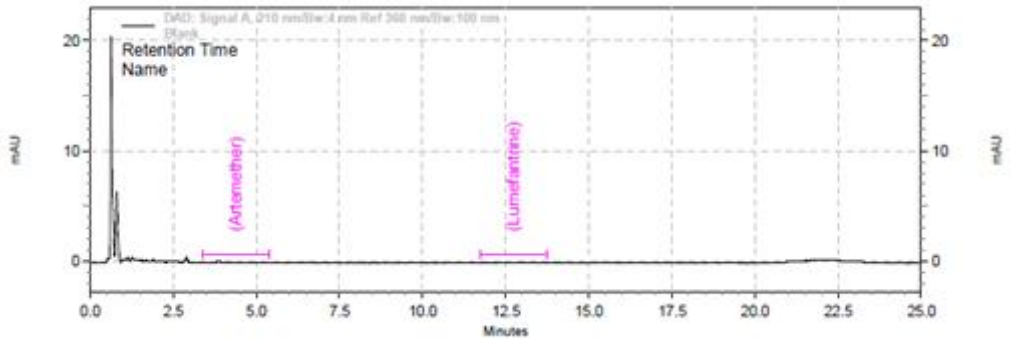
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	280989	9063	1.00	**0.00000**
2	Lumefantrine	12.24	4670549	4094	2.32	**16.77023**

Method Validation

SPECIFICITY
(Blank, Standard & Sample)

Chromatogram No – 7.4

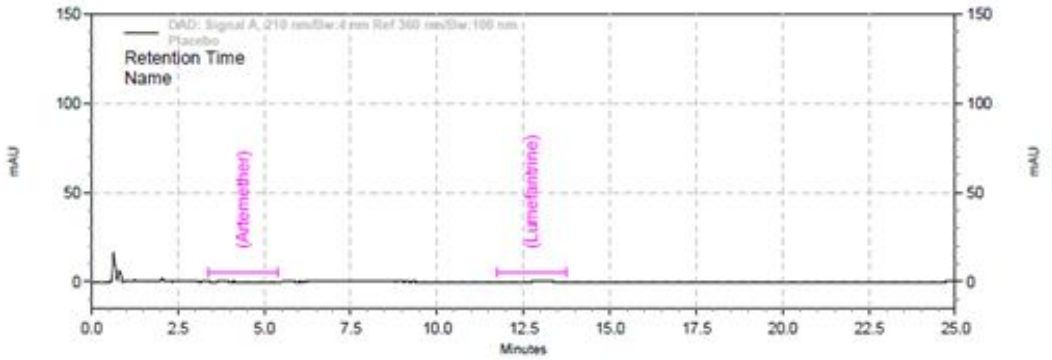
Blank



Peak Number	Name	Area	Retention Time	Theoretical plates (USP)	Asymmetry	Peak purity

Chromatogram No 7.5

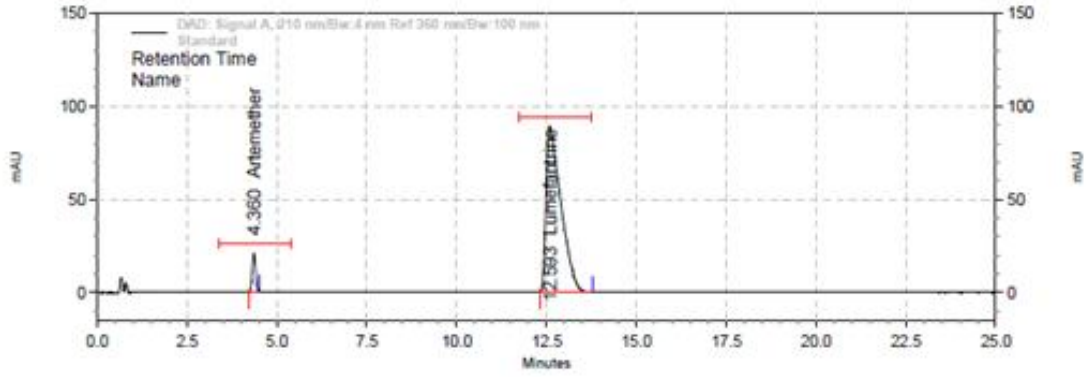
Placebo



Peak Number	Name	Area	Retention Time	Theoretical plates (USP)	Asymmetry	Peak purity

Chromatogram No – 7.6

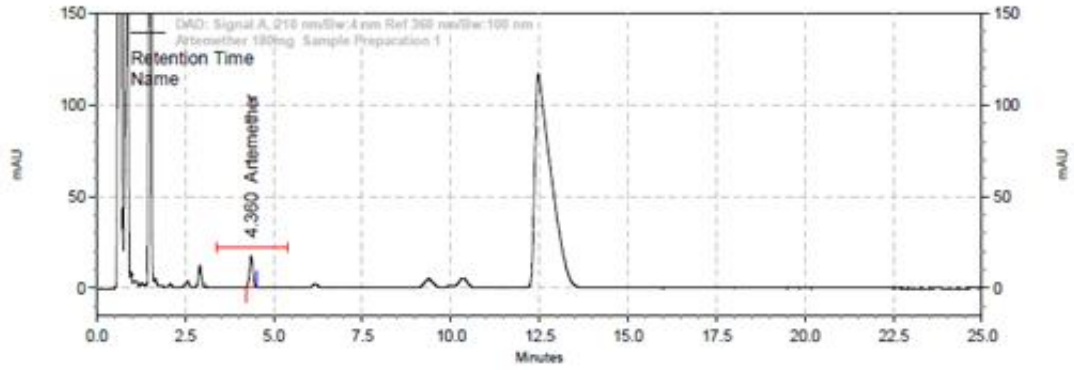
Standard



Peak Number	Name	Area	Retention Time	Theoretical plates (USP)	Asymmetry	Peak purity
1	Artemether	289180	4.36	9380	1.04	**1.000000**
2	Lumefantrine	4972130	12.59	3836	2.33	**1.000000**

Chromatogram No – 7.7

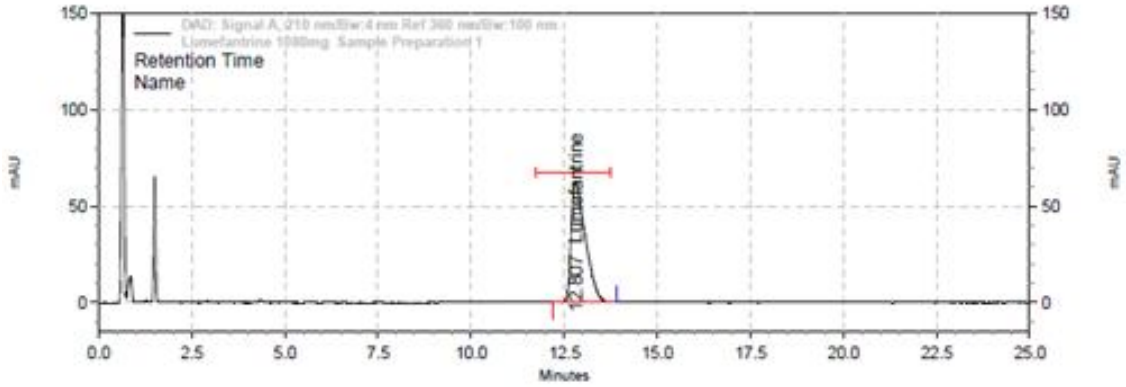
Arthemether Sample Preparation



Peak Number	Name	Area	Retention Time	Theoretical plates (USP)	Asymmetry	Peak purity
1	Artemether	286806	4.36	9510	1.03	1.000000

Chromatogram No – 7.8

Lumefantrine Sample Preparation



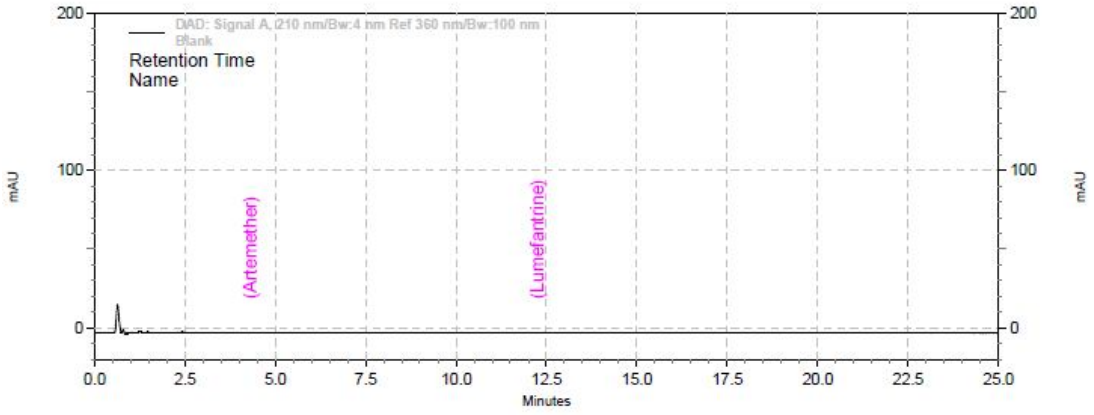
Peak Number	Name	Area	Retention Time	Theoretical plates (USP)	Asymmetry	Peak purity
1	Lumefantrine	4464462	12.81	4944	1.92	1.000000

LINEARITY AND RANGE

(Blank, Standards, linearity samples 60% ,80%,100%,120%,160%)

Chromatogram No – 7.9

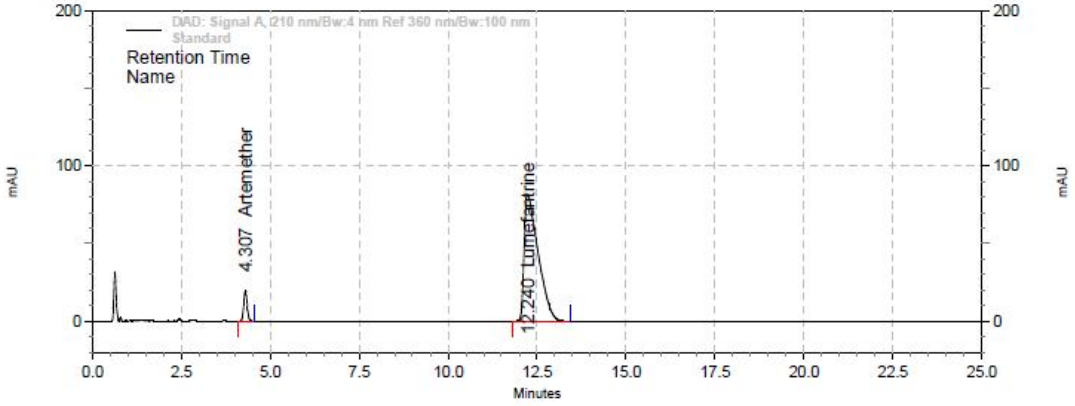
Blank



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry

Chromatogram No – 7.10

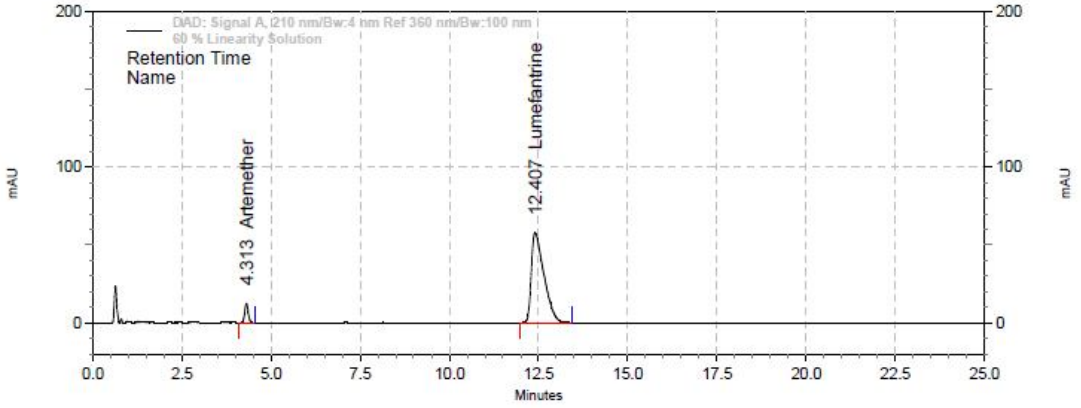
Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	280989	9063	1.00	**0.00**
2	Lumefantrine	12.24	4670549	4094	2.32	**16.77**

Chromatogram No – 7.11

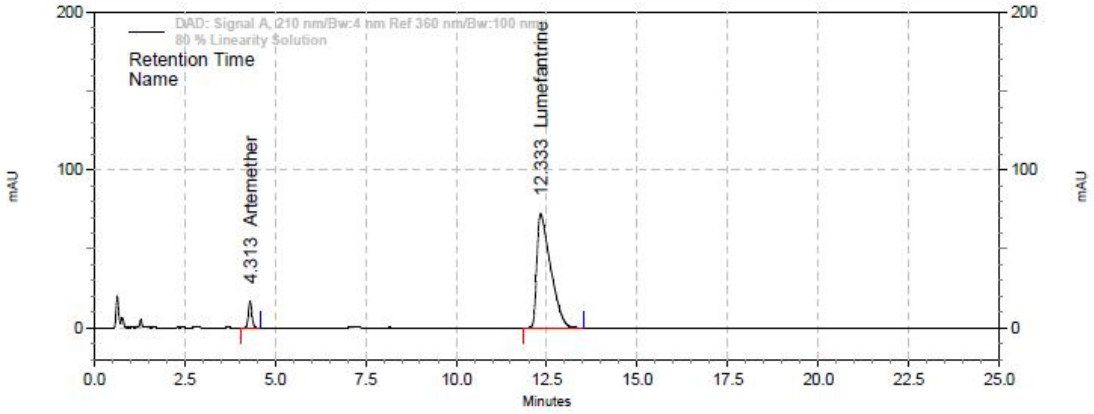
Linearity 60%



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	179294	9062	1.02	**0.00**
2	Lumefantrine	12.41	2979334	5301	1.90	**18.76**

Chromatogram No – 7.12

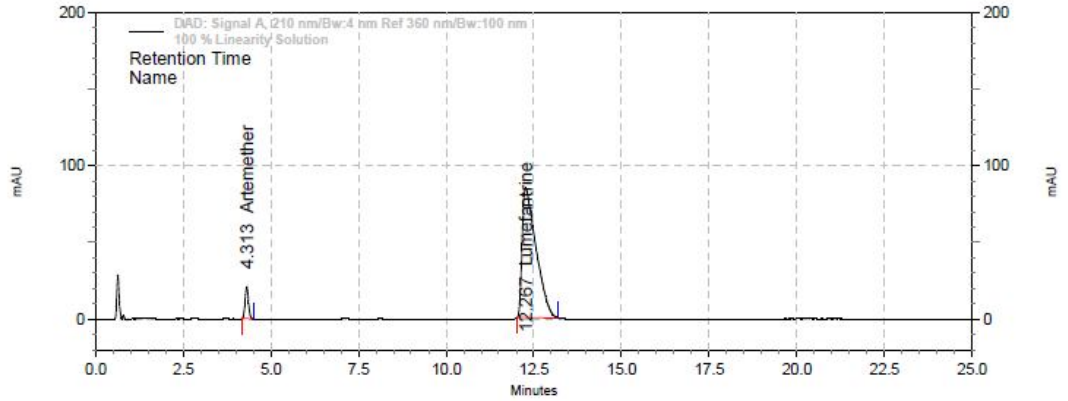
Linearity 80%



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	237715	9072	1.00	**0.00**
2	Lumefantrine	12.33	3983265	4523	2.15	**17.54**

Chromatogram No – 7.13

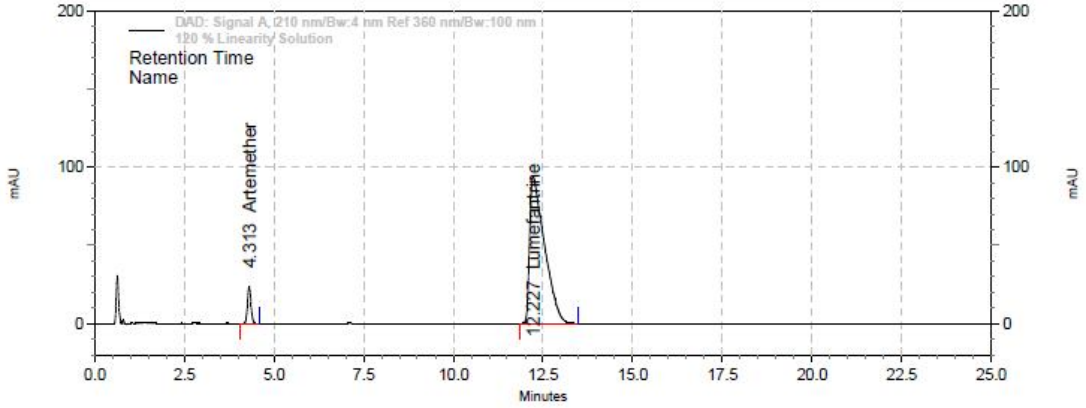
Linearity 100%



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	290764	9307	1.04	**0.00**
2	Lumefantrine	12.27	4876621	3989	2.41	**16.64**

Chromatogram No – 7.14

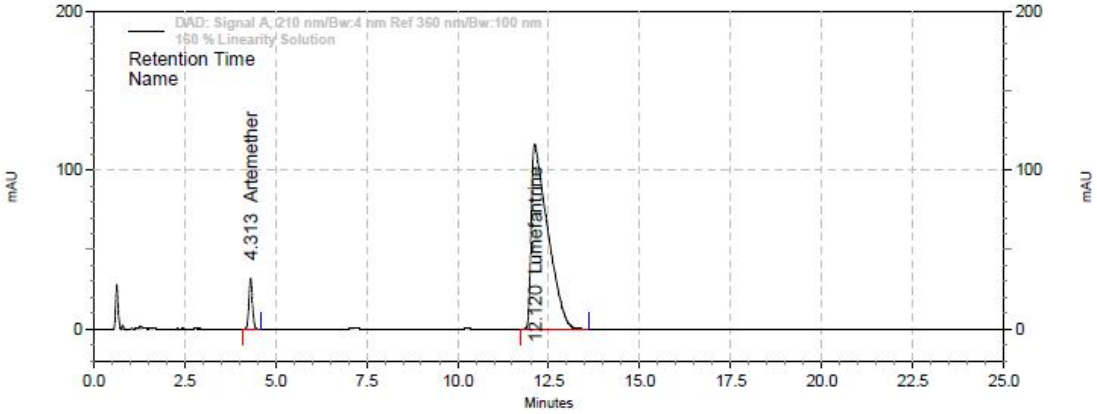
Linearity 120%



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	339031	9104	1.00	**0.00**
2	Lumefantrine	12.23	5662400	3588	2.53	**15.87**

Chromatogram No – 7.15

Linearity 160%



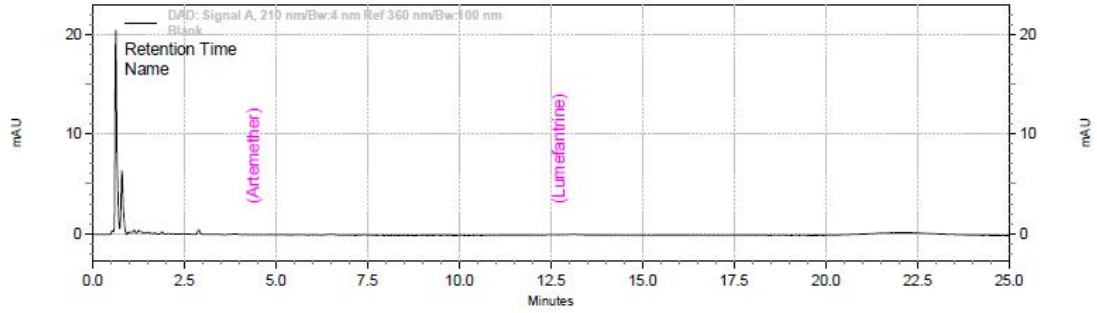
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	455796	9063	1.05	**0.00**
2	Lumefantrine	12.12	7694804	2860	2.84	**14.35**

PRECISION-SYSTEM PRECISION

(Blank and standards)

Chromatogram No – 7.16

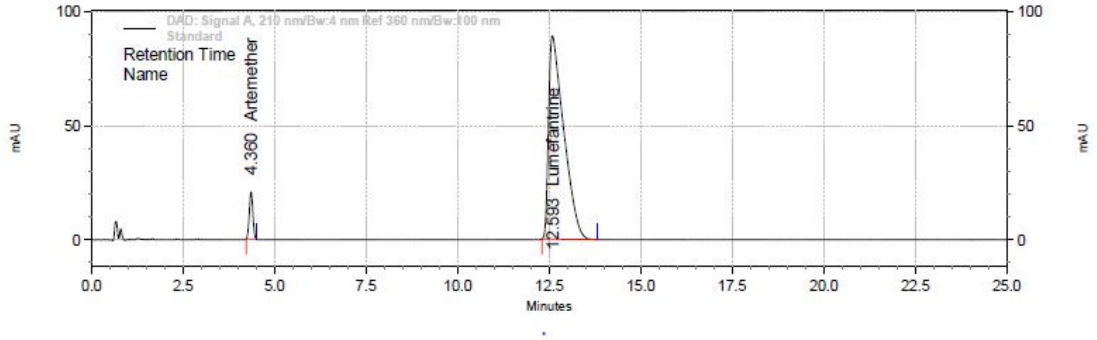
Blank



Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area

Chromatogram No – 7.17

System precision



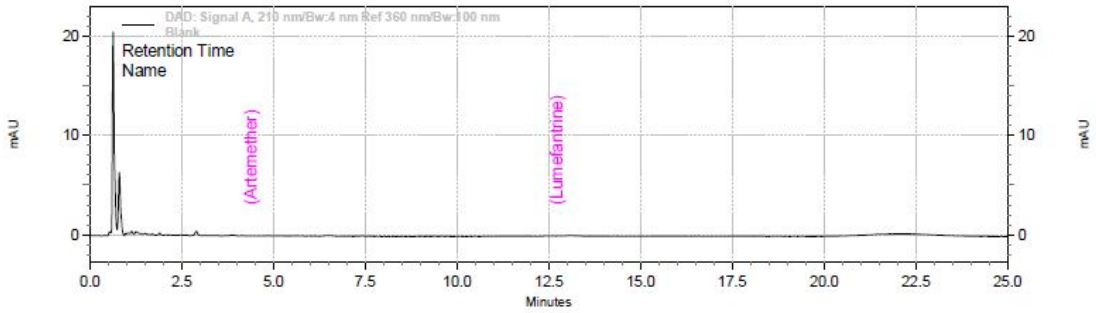
Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area
1	Artemether	4.36	9380	1.04	**0.00**	289180
2	Lumefantrine	12.59	3836	2.33	**16.58**	4972130

PRECISION-METHOD PRECISION

(Blank, standards, Sample preparations)

Chromatogram No – 7.18

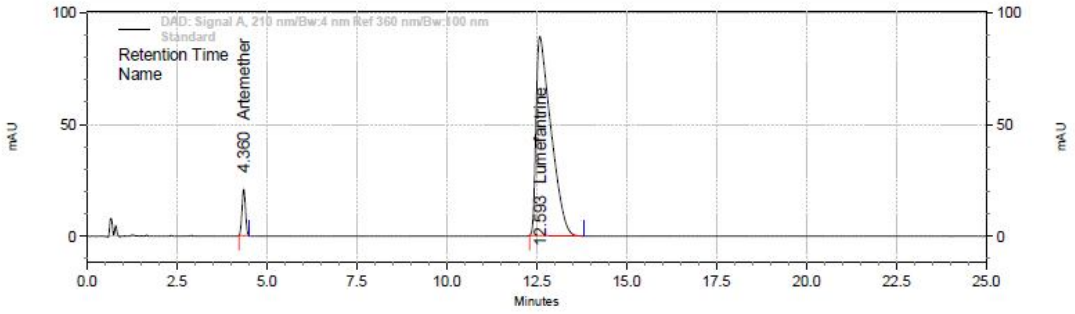
Blank



Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area

Chromatogram No – 7.19

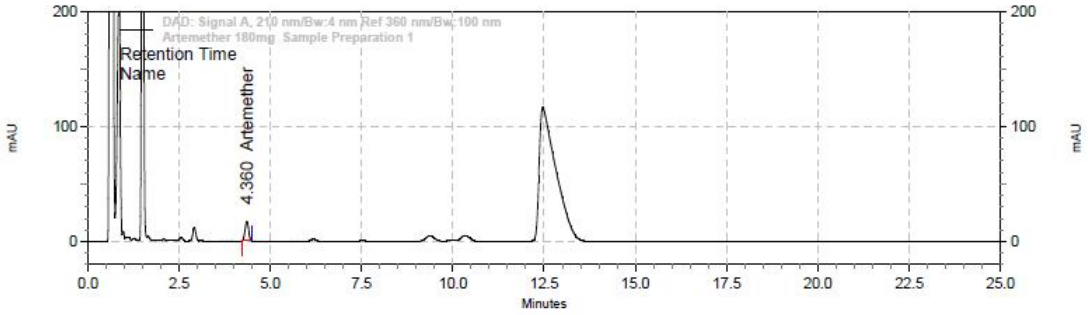
Standard



Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area
1	Artemether	4.36	9380	1.04	**0.00**	289180
2	Lumefantrine	12.59	3836	2.33	**16.58**	4972130

Chromatogram No – 7.20

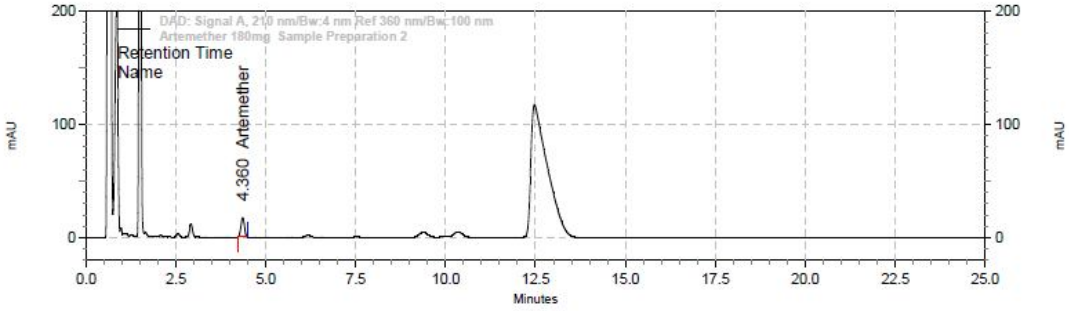
Artemether Sample Preparation - 01



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.36	286806	9510	1.03

Chromatogram No – 7.21

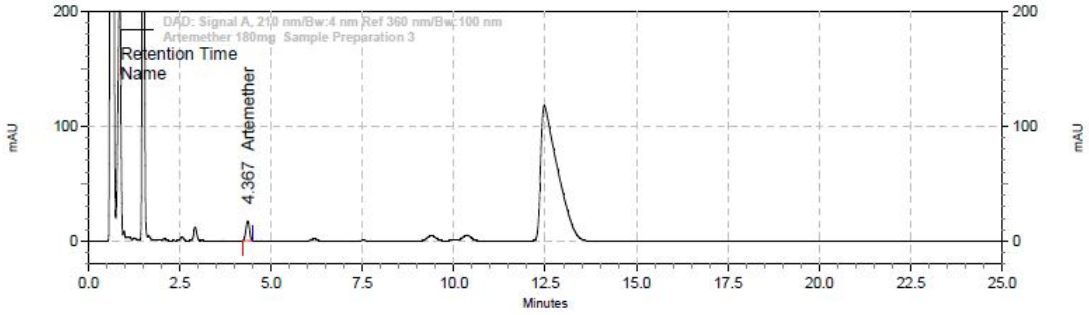
Artemether sample preparation – 02



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.36	285326	9514	1.04

Chromatogram No – 7.22

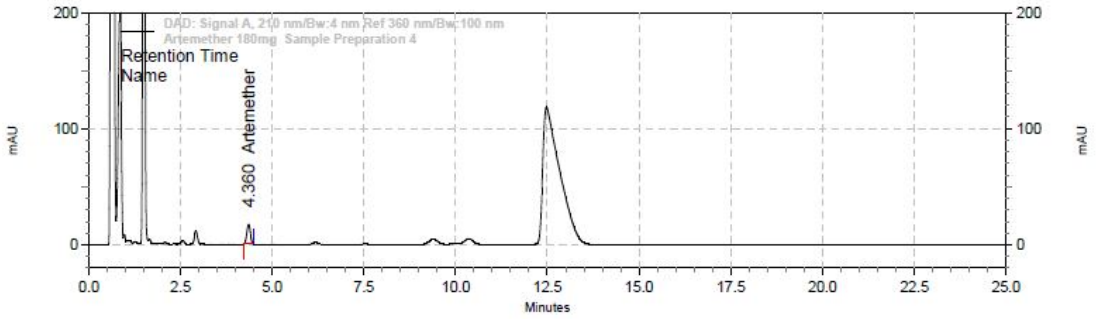
Artemether sample preparation -03



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.37	286393	9525	0.99

Chromatogram No – 7.23

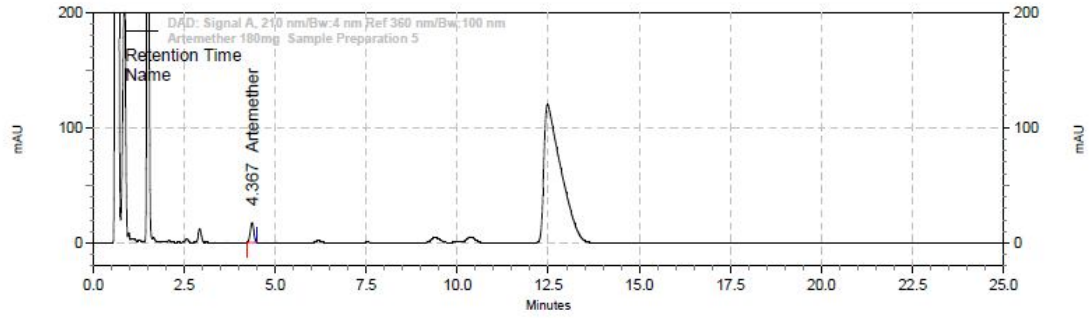
Artemether sample preparation -04



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.36	285938	9518	1.05

Chromatogram No – 7.24

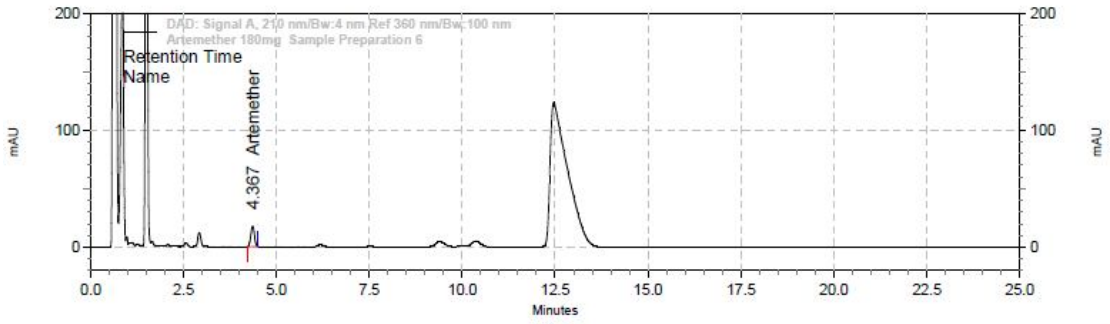
Artemether sample preparation -05



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.37	287828	9538	1.02

Chromatogram No – 7.25

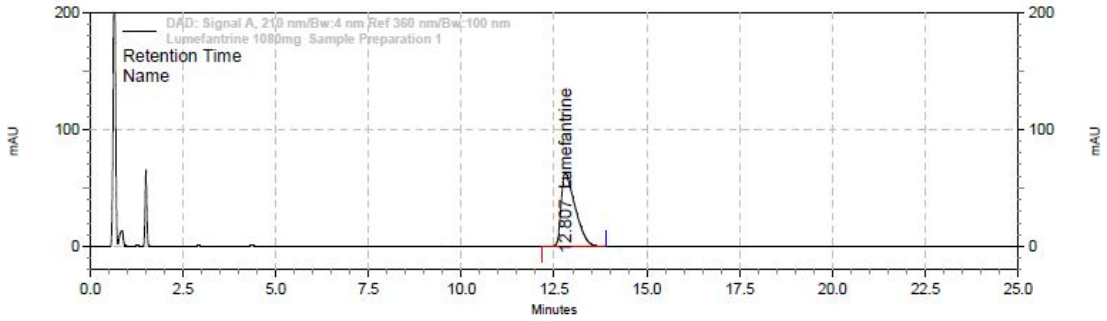
Artemether sample preparation - 06



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.37	289119	9511	1.00

Chromatogram No – 7.26

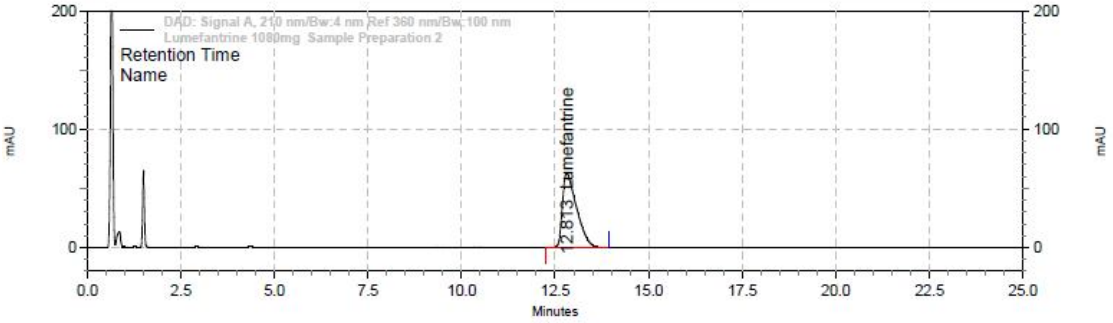
Lumefantrine Sample Preparation - 01



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.81	4464462	4944	1.92

Chromatogram No – 7.27

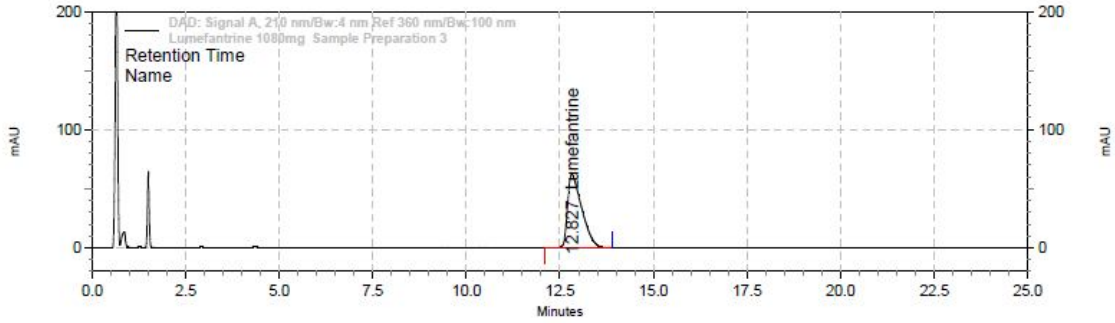
Lumefantrine Sample Preparation - 02



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.81	4456902	4933	1.92

Chromatogram No – 7.28

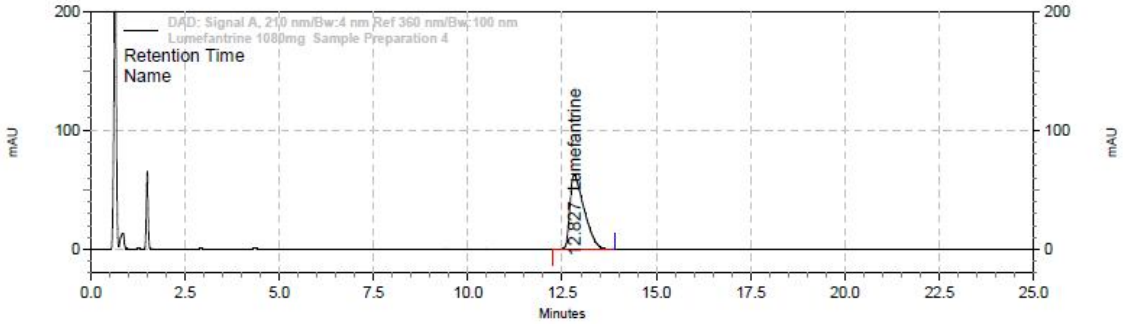
Lumefantrine Sample Preparation - 03



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.83	4429942	4971	1.89

Chromatogram No – 7.29

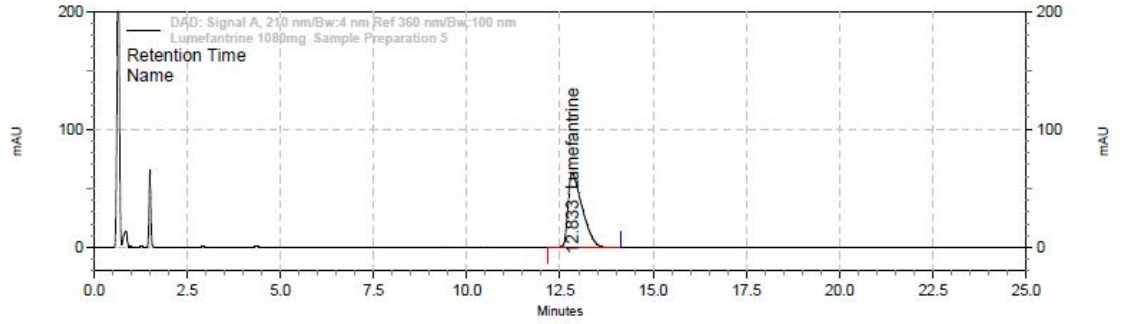
Lumefantrine Sample Preparation - 04



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.83	4456342	4935	1.90

Chromatogram No – 7.30

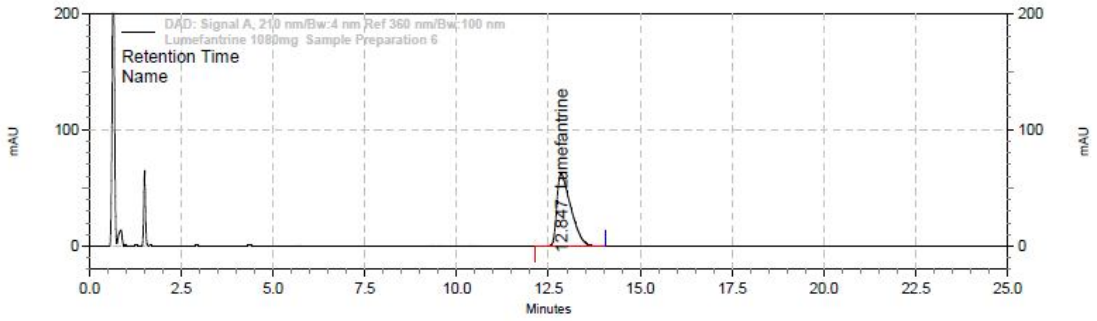
Lumefantrine Sample Preparation - 05



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.83	4489968	4905	1.93

Chromatogram No – 7.31

Lumefantrine Sample Preparation - 06

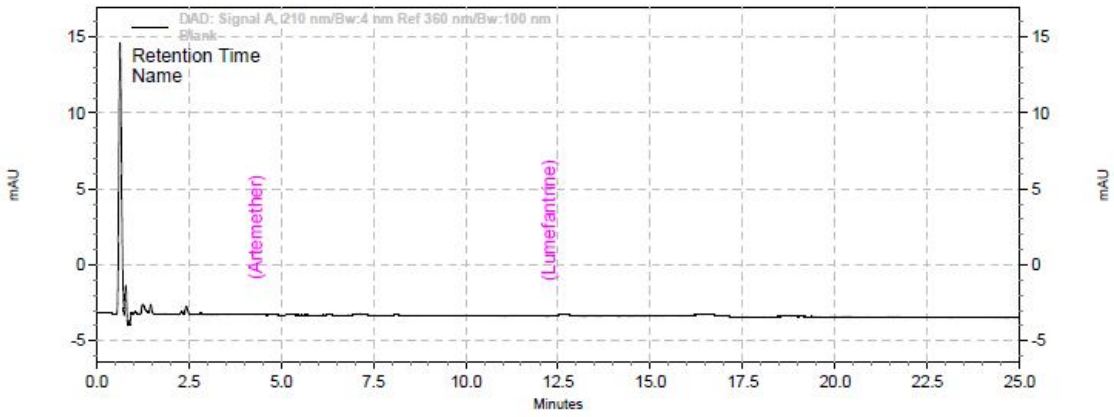


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.85	4441994	4940	1.92

ACCURACY

Chromatogram No – 7.32

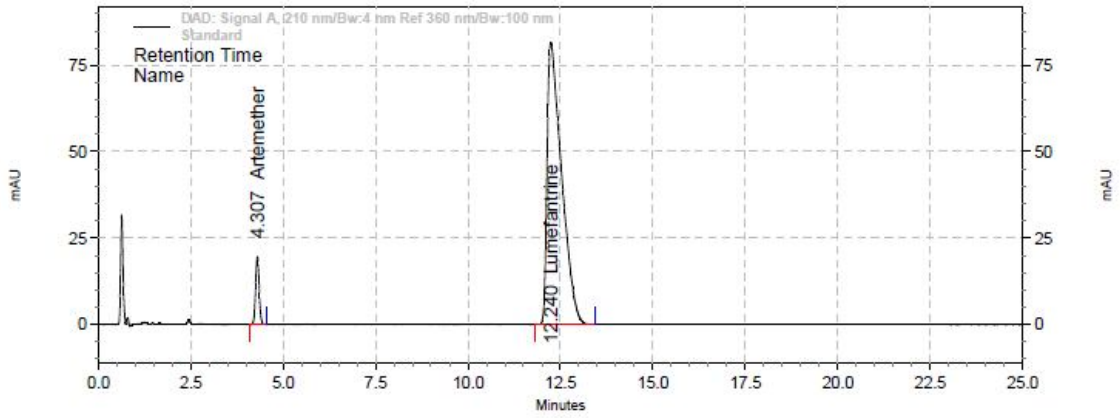
Blank



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry

Chromatogram No – 7.33

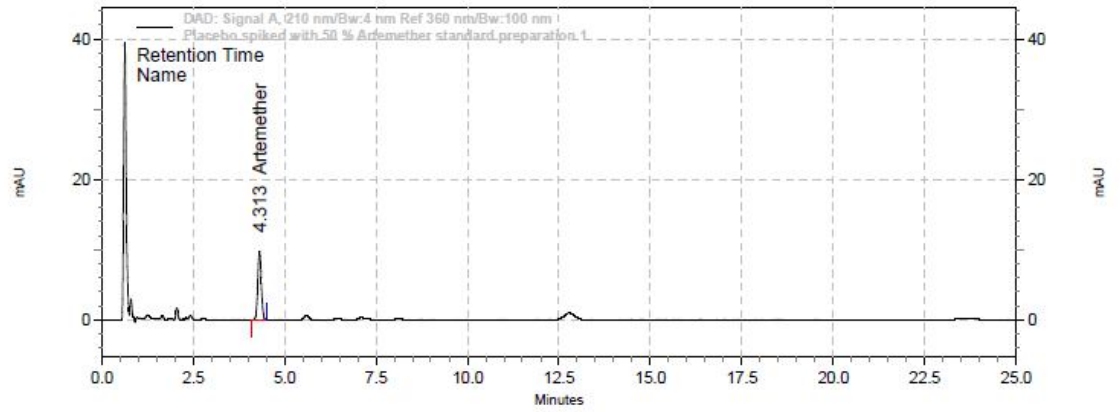
Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.31	280989	9063	1.00	**0.00000**
2	Lumefantrine	12.24	4670549	4094	2.32	**16.77023**

Chromatogram No – 7.34

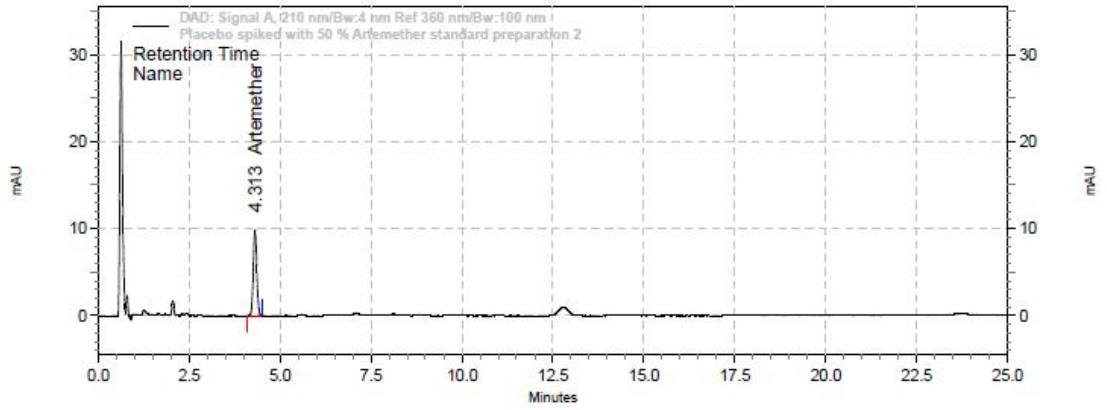
Accuracy 50%- Artemether Preparation-1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.31	140426	9087	1.04

Chromatogram No – 7.35

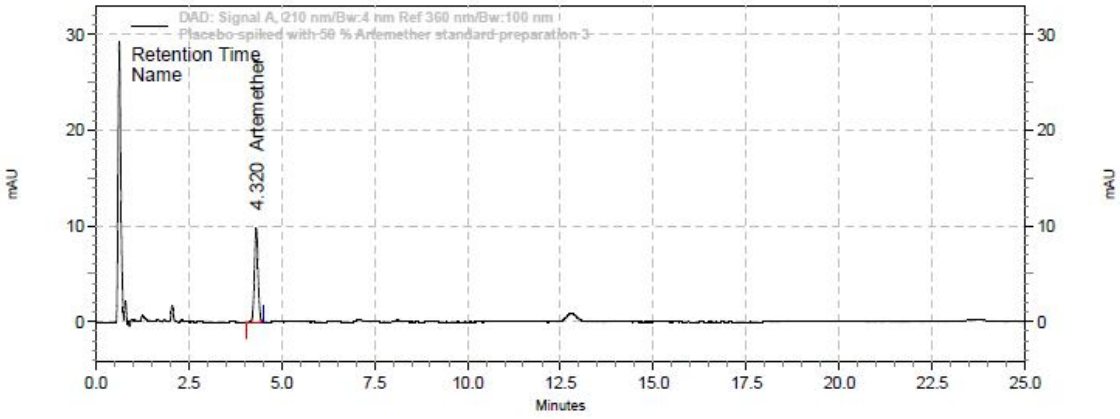
Accuracy 50% - Artemether Preparation-2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.31	141156	9071	1.02

Chromatogram No – 7.36

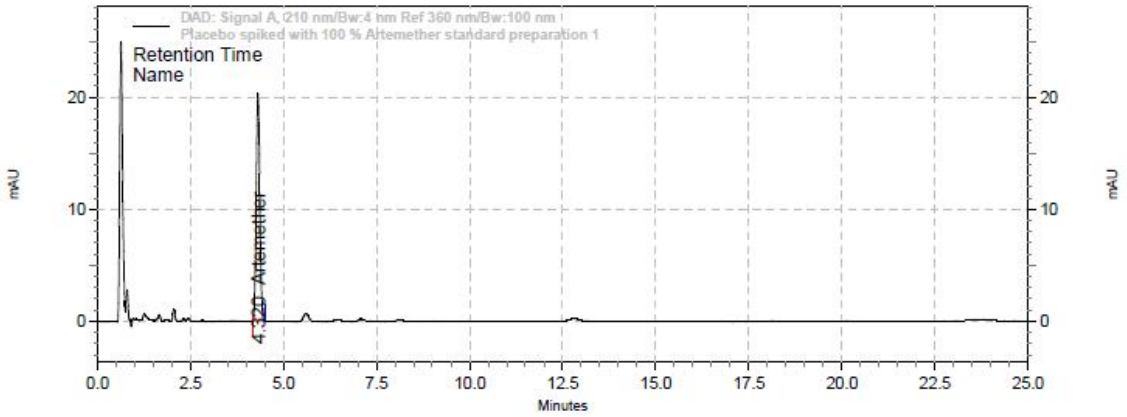
Accuracy 50% - Artemether Preparation-3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.32	140990	9095	1.01

Chromatogram No – 7.37

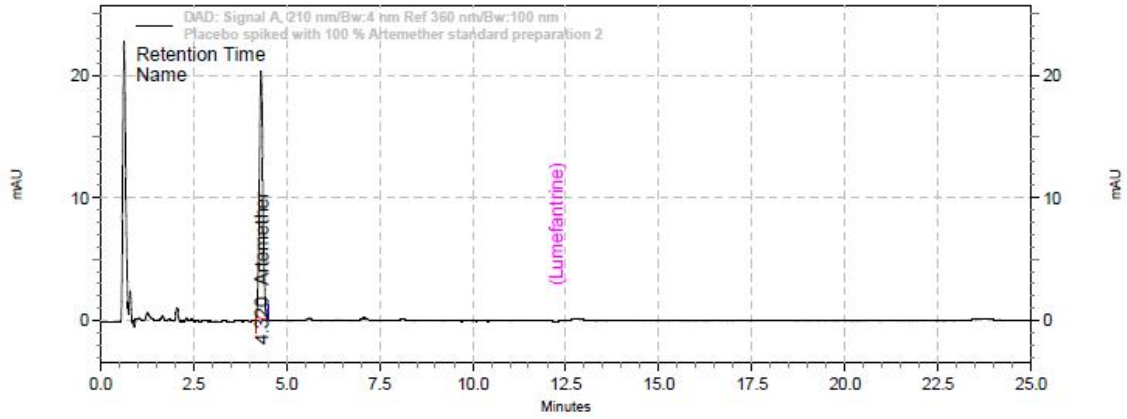
Accuracy 100% - Artemether Preparation-1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.32	284290	9224	1.02

Chromatogram No – 7.38

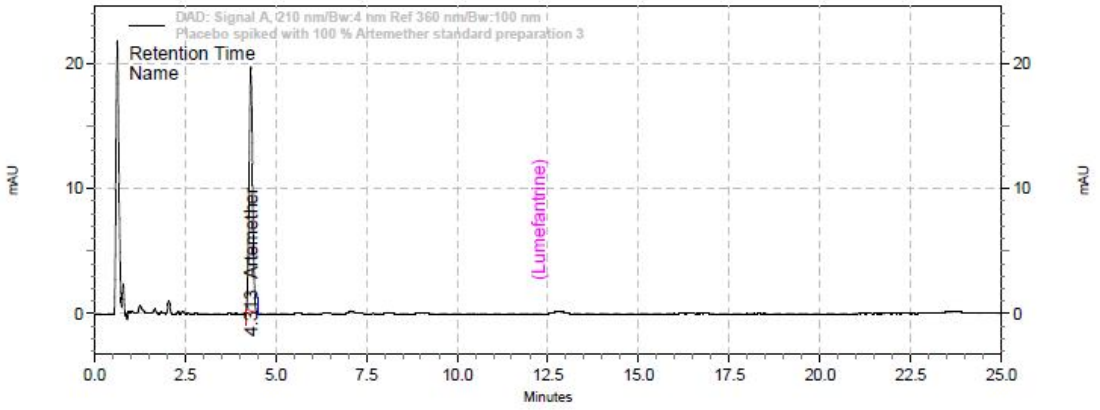
Accuracy 100% -Artemether Preparation-2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.32	284238	9226	1.03

Chromatogram No – 7.39

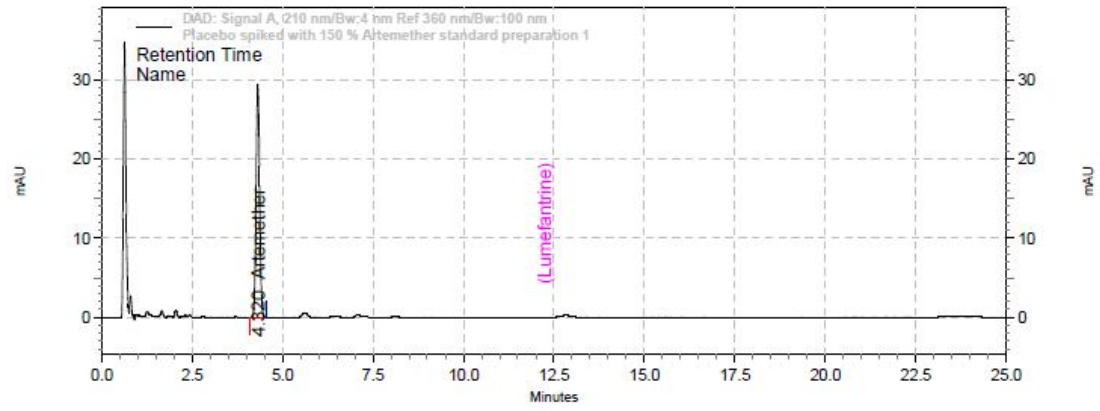
Accuracy 100% - Artemether Preparation-3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.31	275077	9185	1.06

Chromatogram No – 7.40

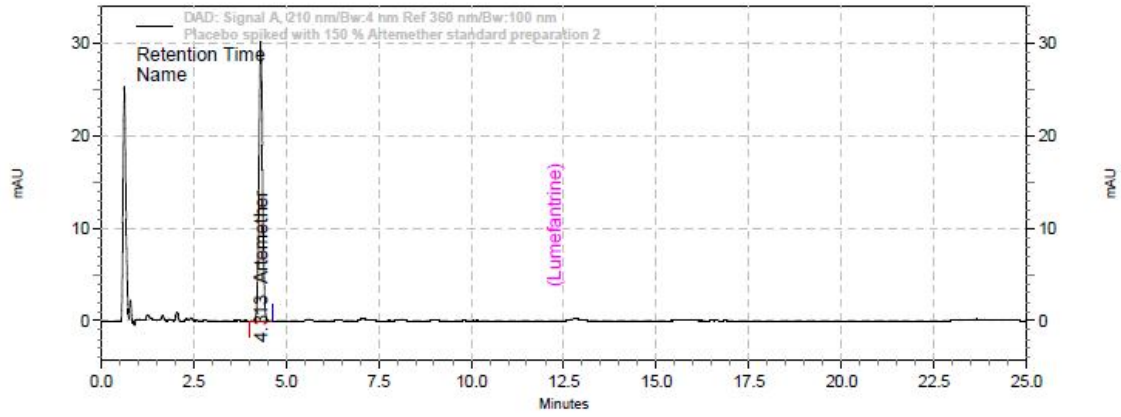
Accuracy 150% - Artemether Preparation-1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.32	422397	9066	1.01

Chromatogram No – 7.41

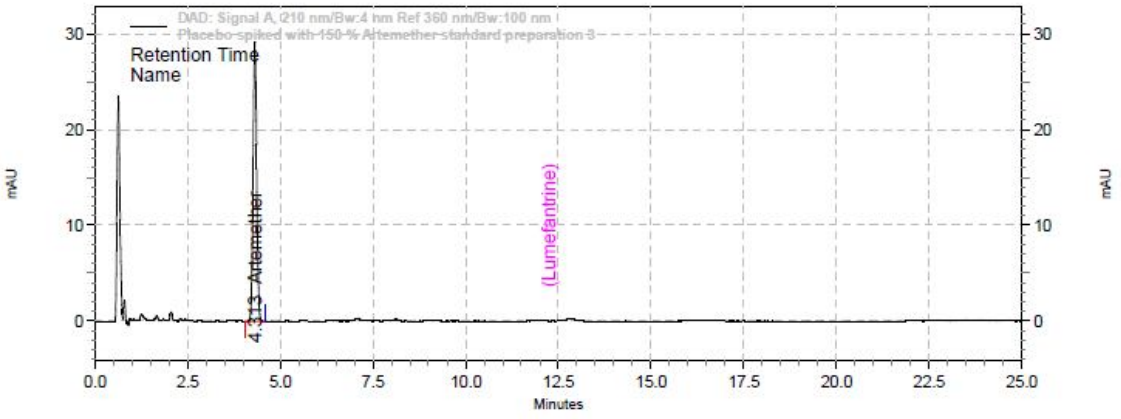
Accuracy 150% - Artemether Preparation-2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.31	434774	9043	1.06

Chromatogram No – 7.42

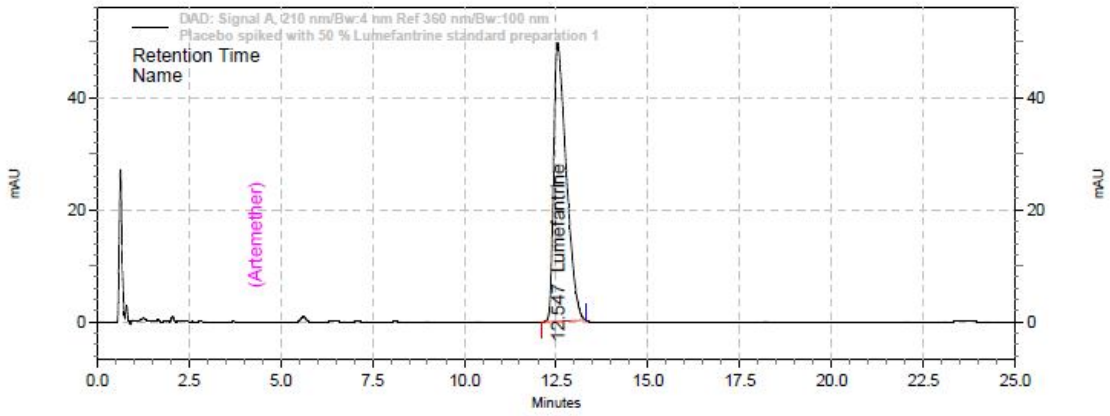
Accuracy 150% - Artemether Preparation-3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.31	420330	9027	1.06

Chromatogram No – 7.43

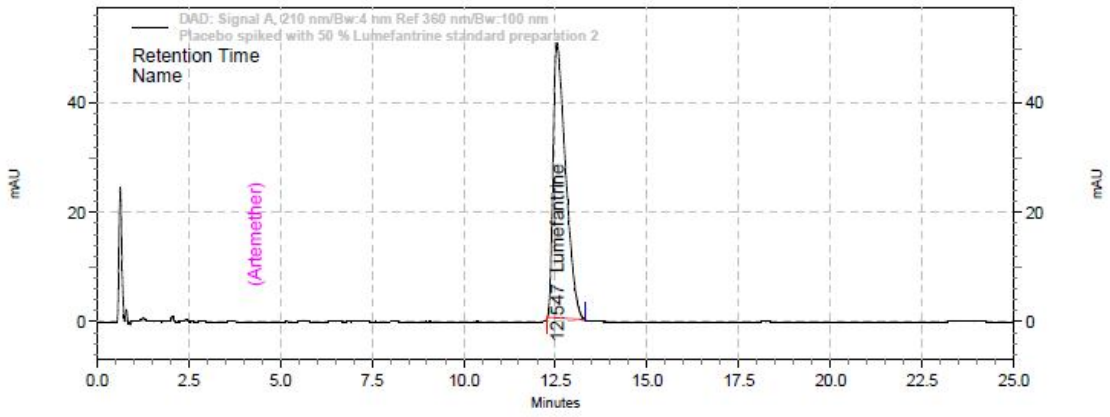
Accuracy 50 %- Lumefantrine Preparation-1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.55	2499091	5743	1.76

Chromatogram No – 7.44

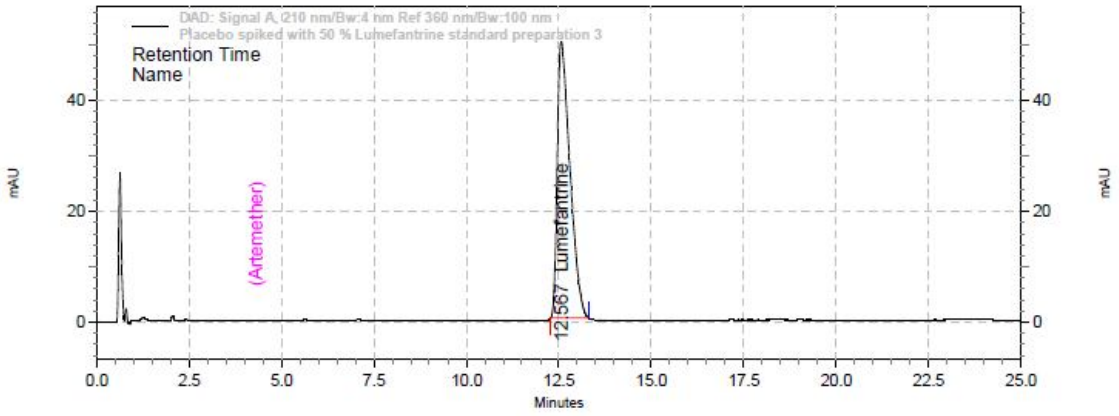
Accuracy 50% - Lumefantrine Preparation-2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.55	2515721	5732	1.84

Chromatogram No – 7.45

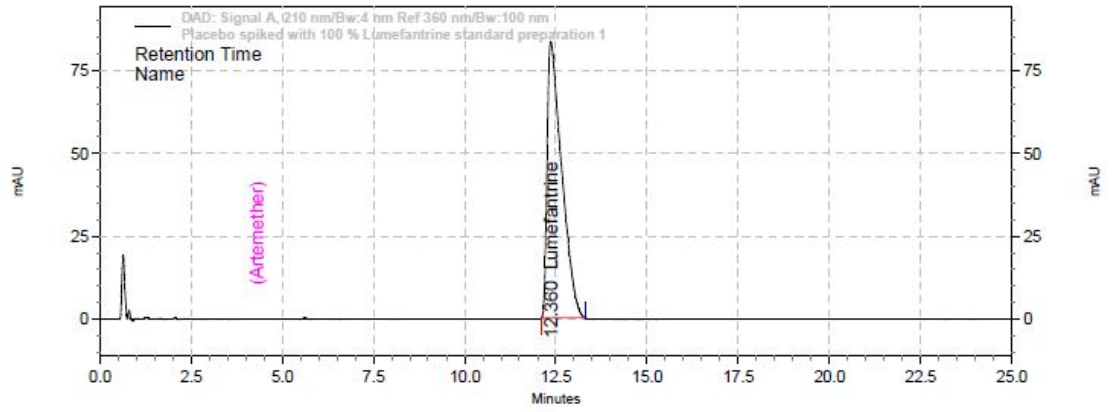
Accuracy 50% - Lumefantrine Preparation-3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.57	2496075	5760	1.83

Chromatogram No – 7.46

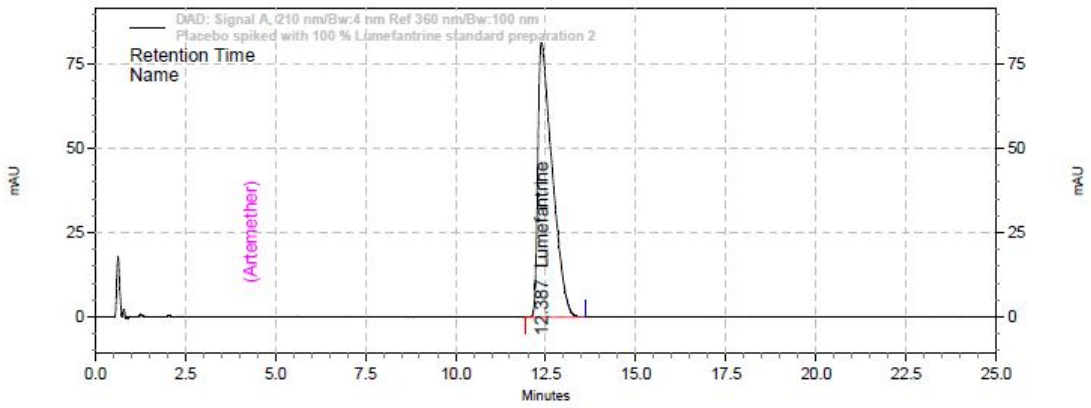
Accuracy 100% - Lumefantrine Preparation-1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.36	4837082	3989	2.41

Chromatogram No – 7.47

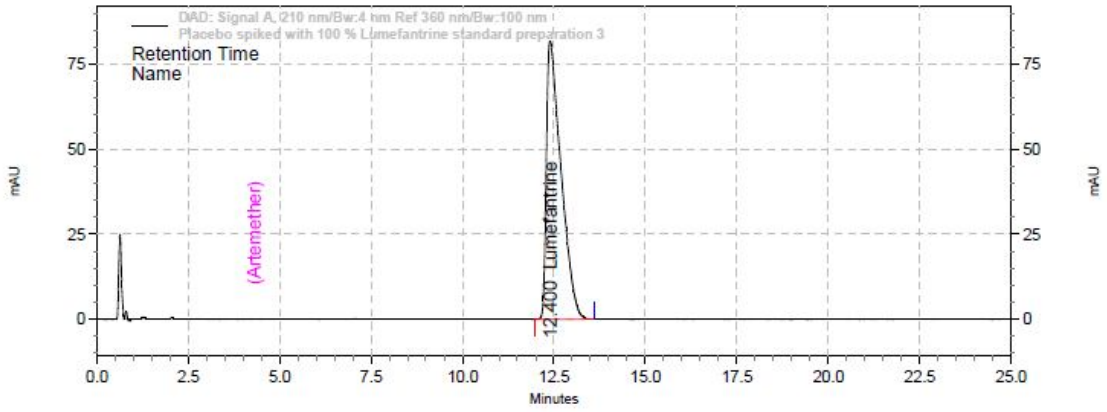
Accuracy 100% - Lumefantrine Preparation-2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.39	4738074	4032	2.33

Chromatogram No – 7.48

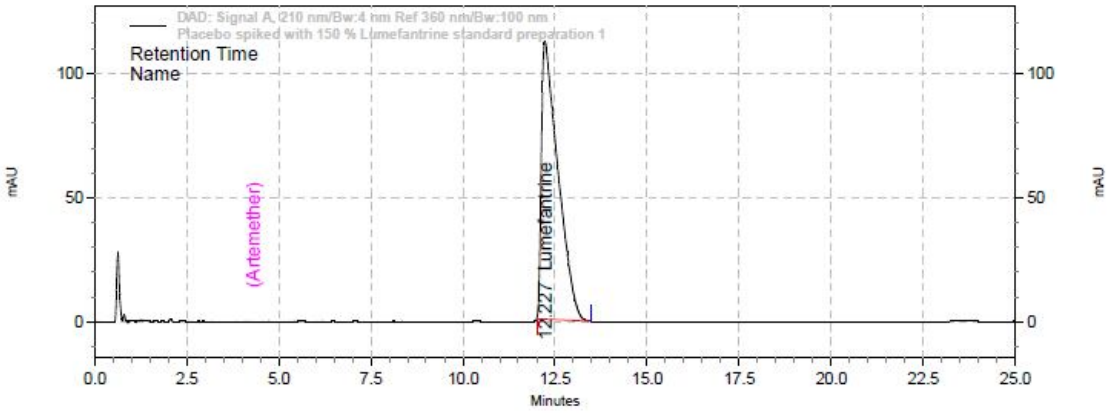
Accuracy 100% - Lumefantrine Preparation-3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.40	4771328	4013	2.31

Chromatogram No – 7.49

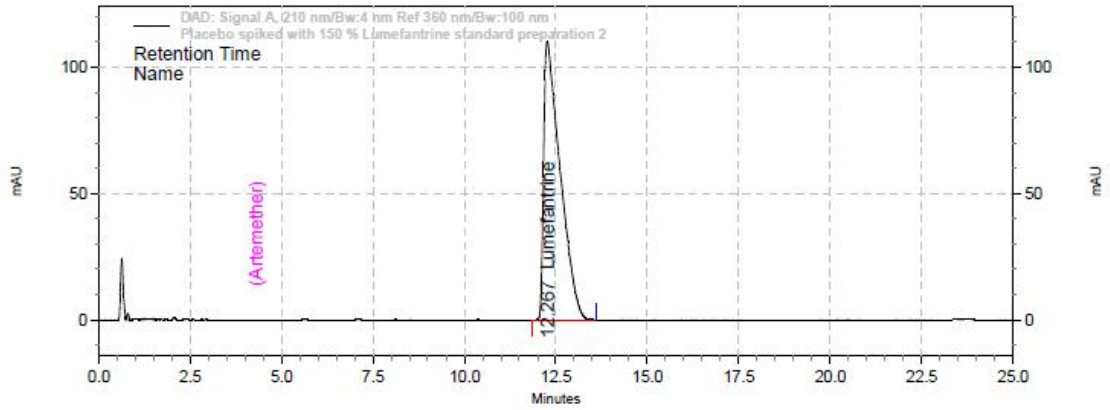
Accuracy 150 % - Lumefantrine Preparation-1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.23	7358306	2933	2.91

Chromatogram No – 7.50

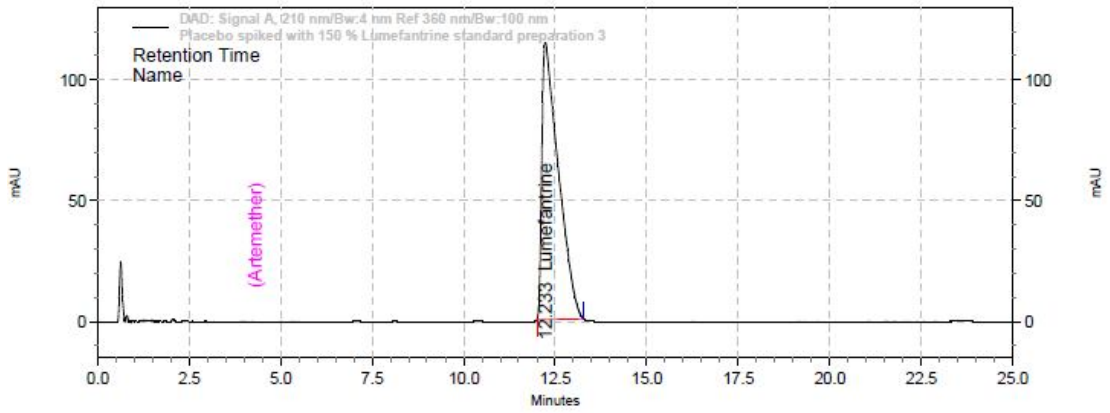
Accuracy 150% - Lumefantrine Preparation-2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.27	7245548	2978	2.83

Chromatogram No – 7.51

Accuracy 150% - Lumefantrine Preparation-3



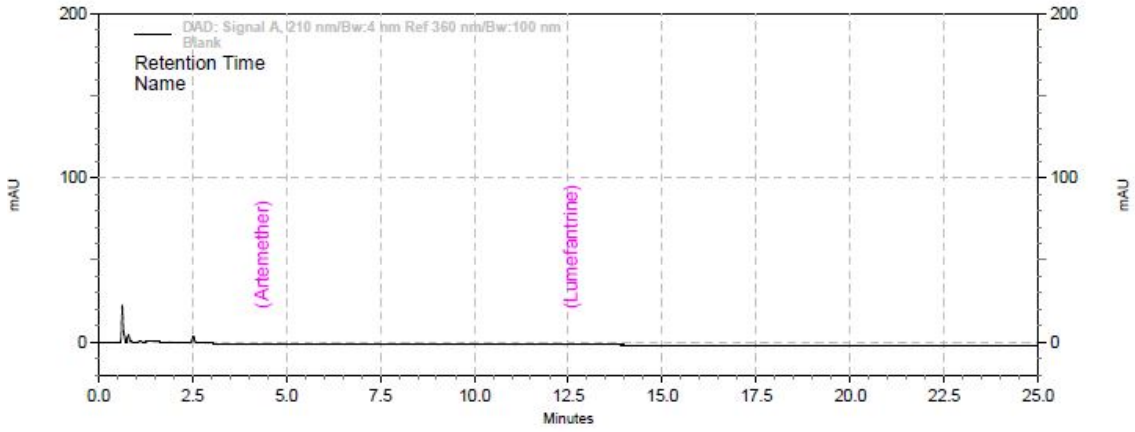
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.23	7545683	2893	2.89

RUGGEDNESS (INTERMEDIATE PRECISION)

(Blank ,standards, Sample preparations)

Chromatogram No – 7.52

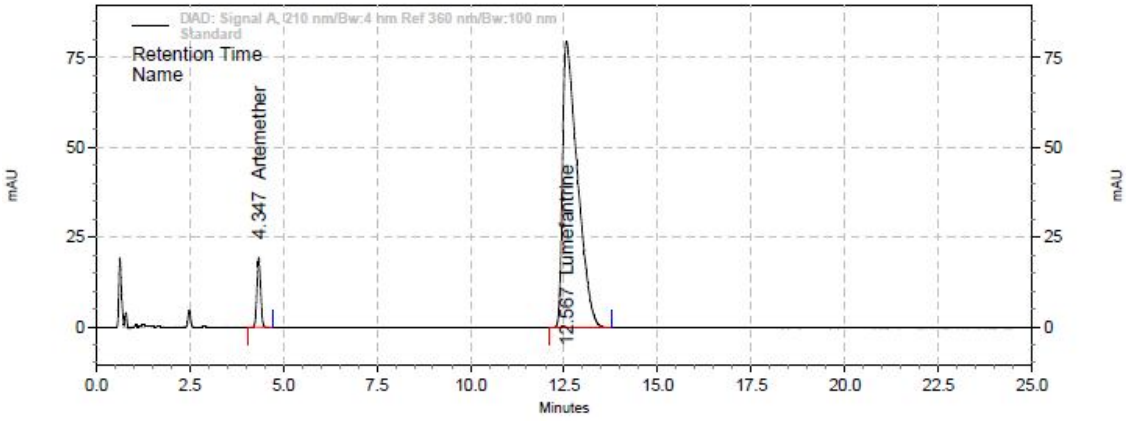
Blank



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)

Chromatogram No – 7.53

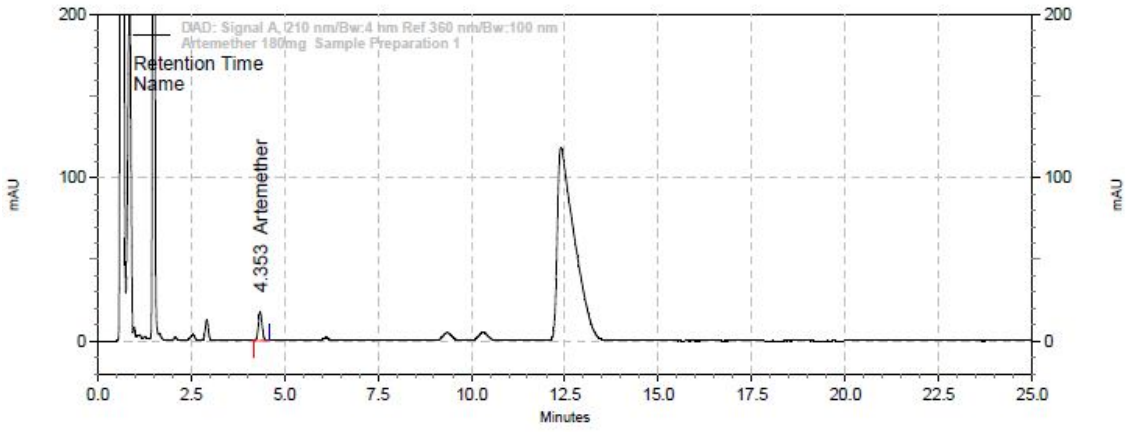
Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	280281	9053	1.01	**0.00000**
2	Lumefantrine	12.57	4651715	4163	2.19	**17.09351**

Chromatogram No – 7.54

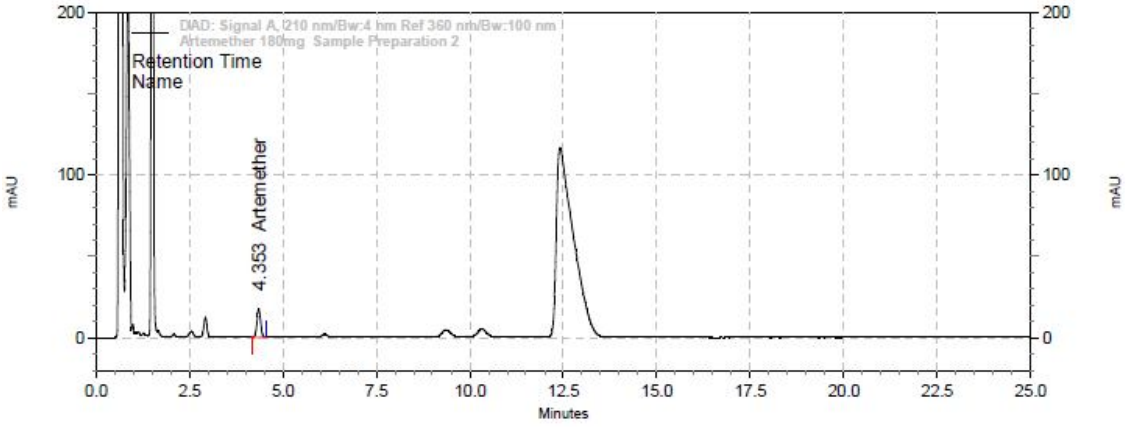
Artemether Sample Preparation – 1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	273295	9357	1.01

Chromatogram No – 7.55

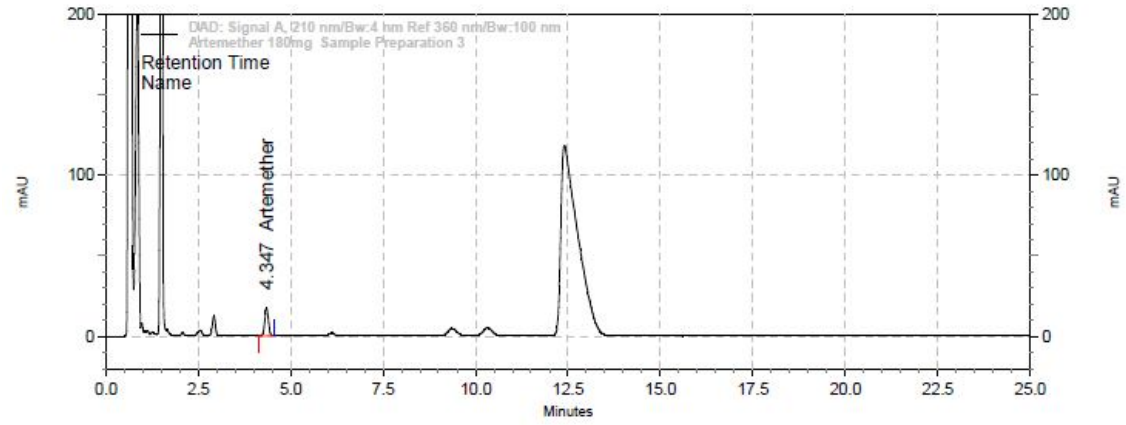
Artemether Sample Preparation - 2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	277150	9329	1.04

Chromatogram No – 7.56

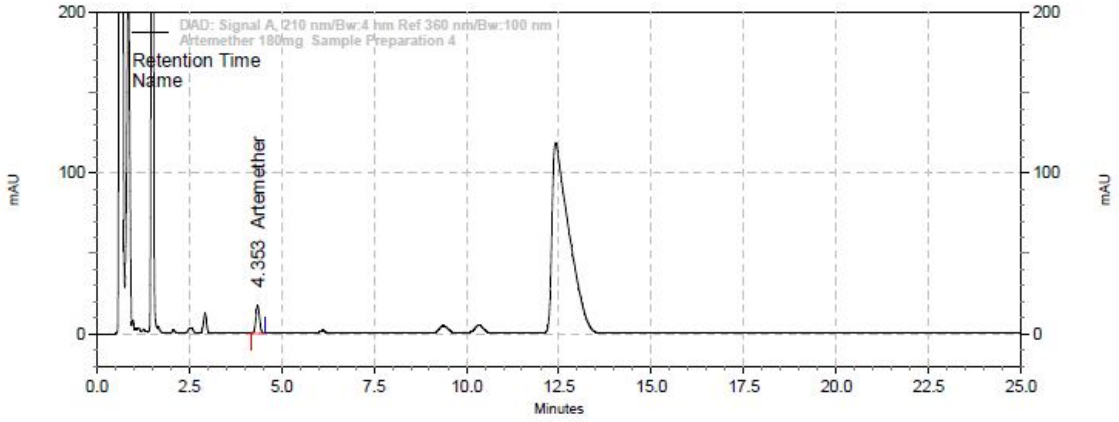
Artemether Sample Preparation - 3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	278465	9360	1.01

Chromatogram No – 7.57

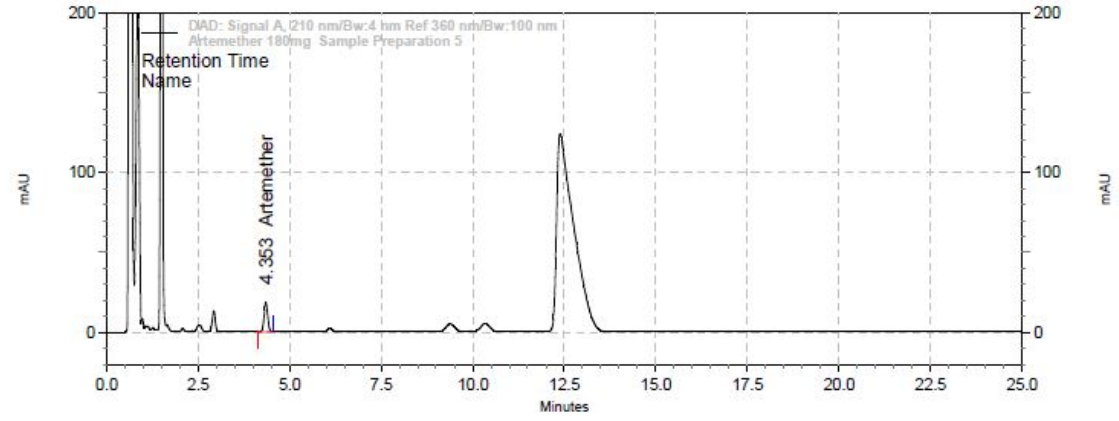
Artemether Sample Preparation - 4



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	278493	9326	1.03

Chromatogram No – 7.58

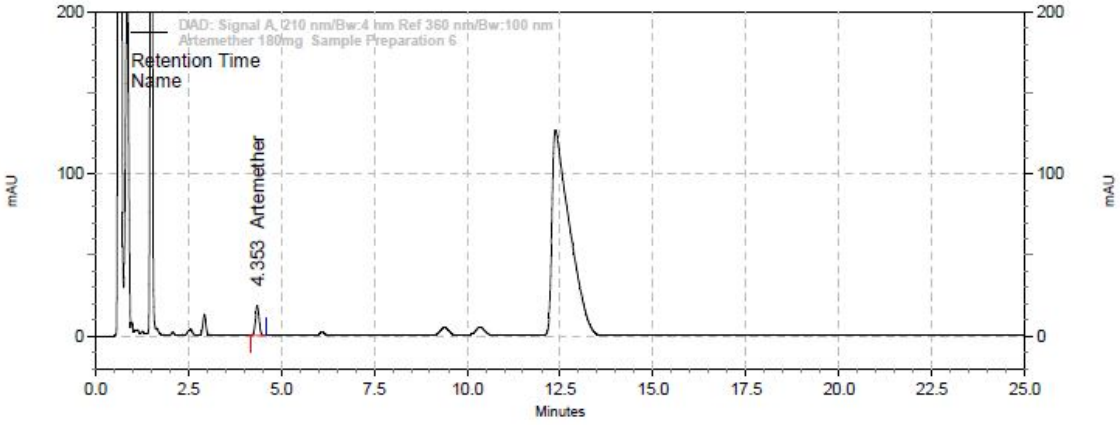
Artemether Sample Preparation - 5



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	270769	9373	1.03

Chromatogram No – 7.59

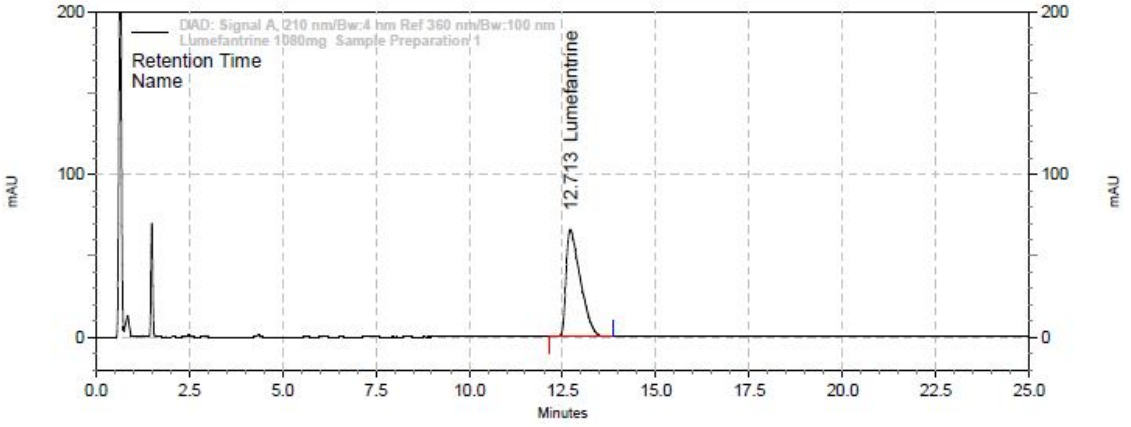
Artemether Sample Preparation – 6



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	271712	9343	1.04

Chromatogram No – 7.60

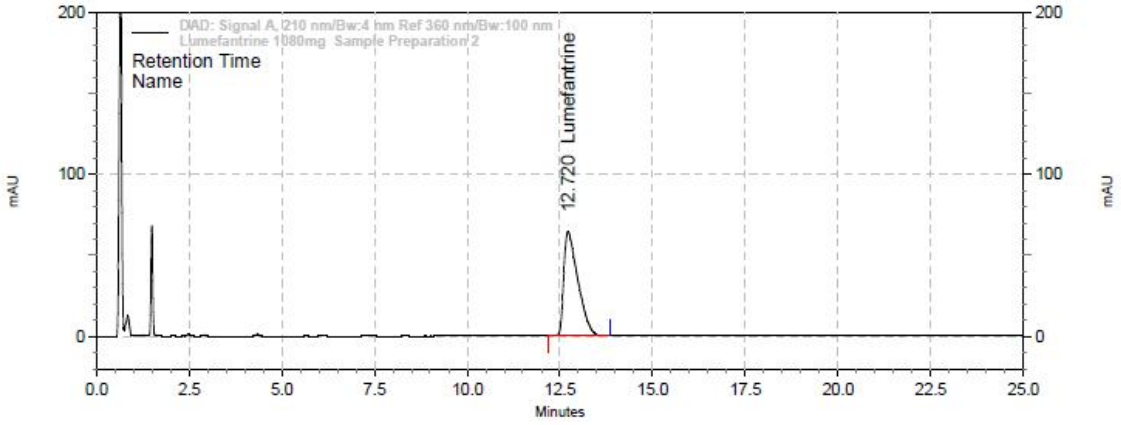
Lumefantrine Sample Preparation - 1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.71	4289102	4747	1.96

Chromatogram No – 7.61

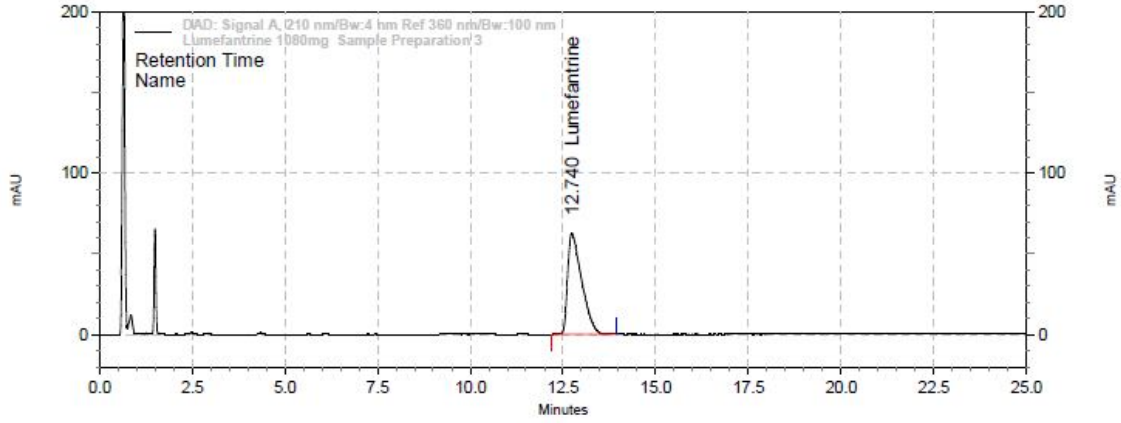
Lumefantrine Sample Preparation - 2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.72	4291352	4798	1.97

Chromatogram No – 7.62

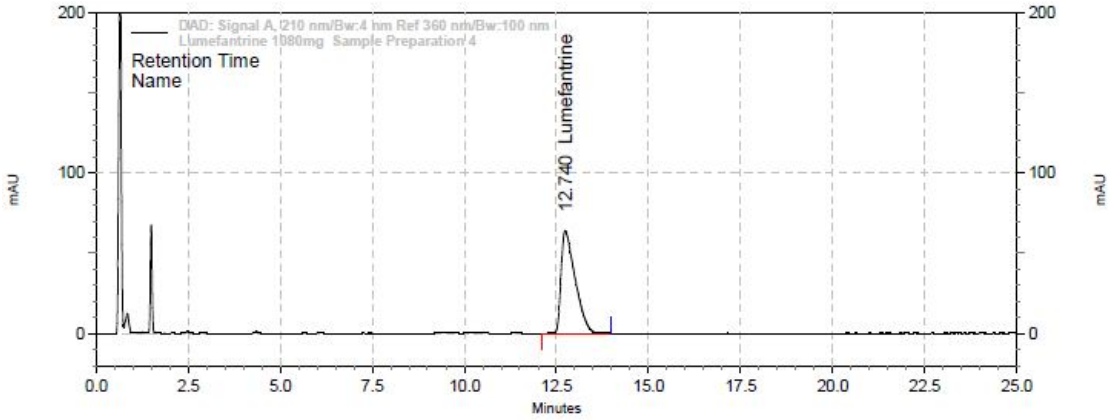
Lumefantrine Sample Preparation - 3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.74	4258195	4894	1.95

Chromatogram No – 7.63

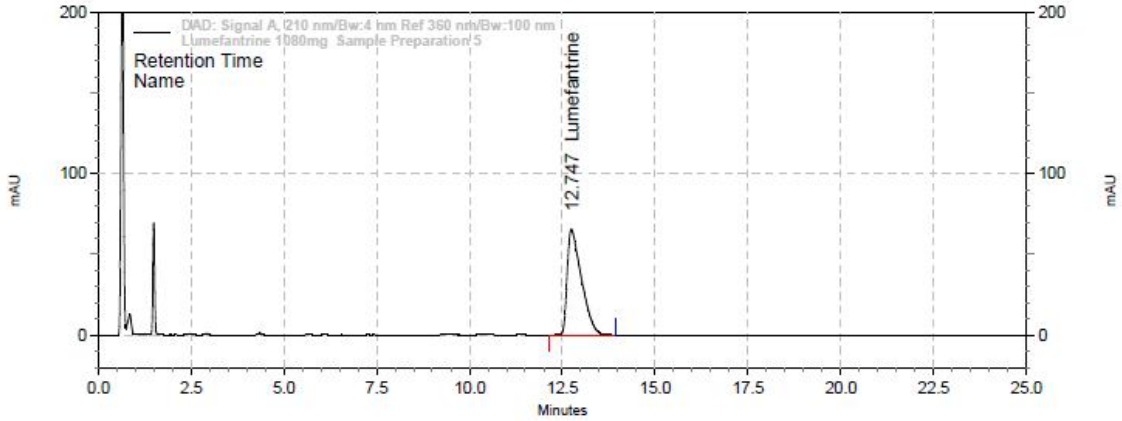
Lumefantrine Sample Preparation - 4



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.74	4260643	4810	1.97

Chromatogram No – 7.64

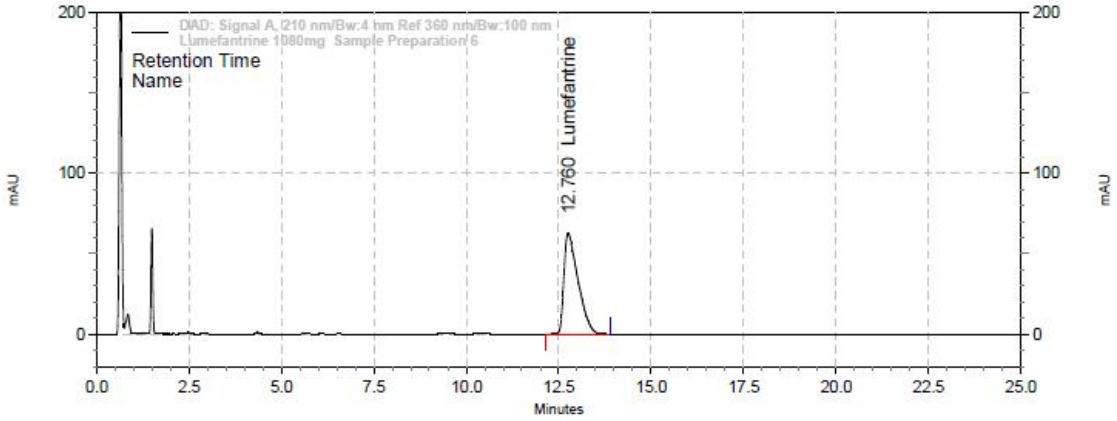
Lumefantrine Sample Prepararion - 5



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.75	4261898	4749	1.99

Chromatogram No – 7.65

Lumefantrine Sample Preparation - 6

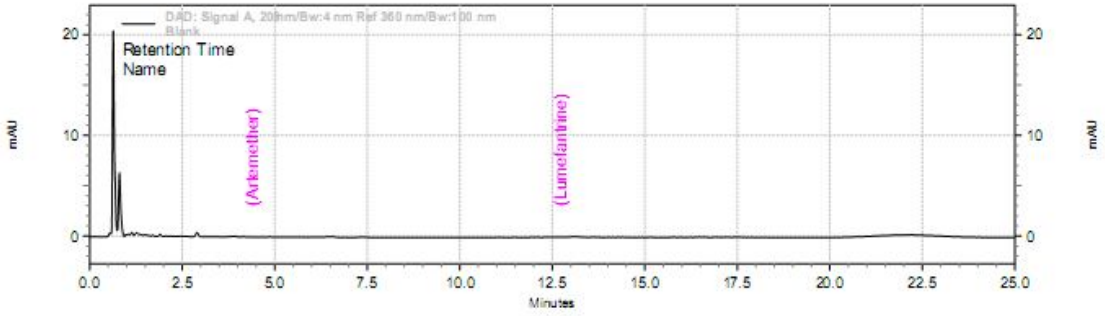


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.76	4280265	4865	1.92

ROBUSTNESS

Chromatogram No – 7.66

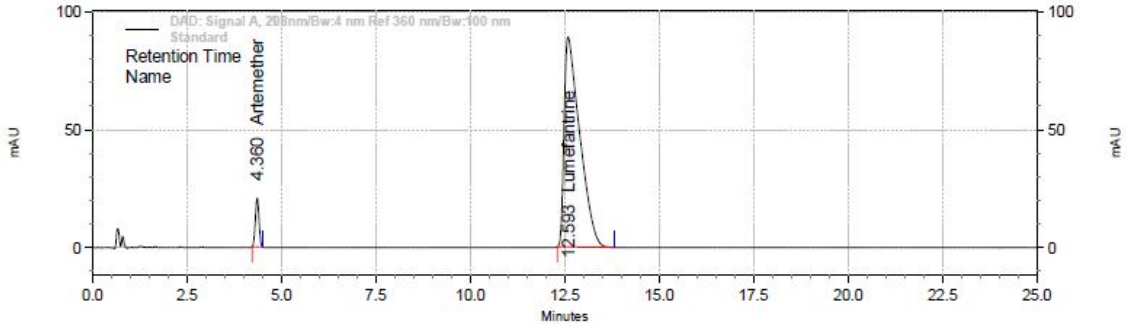
Change in the wave length -208 nm - Blank



Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area

Chromatogram No – 7.67

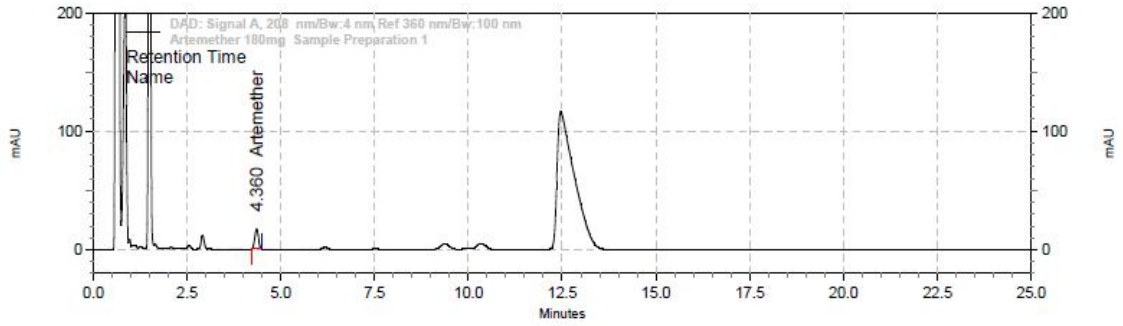
Change in the wave length -208 nm - Standard



Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area
1	Artemether	4.36	9380	1.04	**0.00**	289180
2	Lumefantrine	12.59	3836	2.33	**16.58**	4972130

Chromatogram No – 7.68

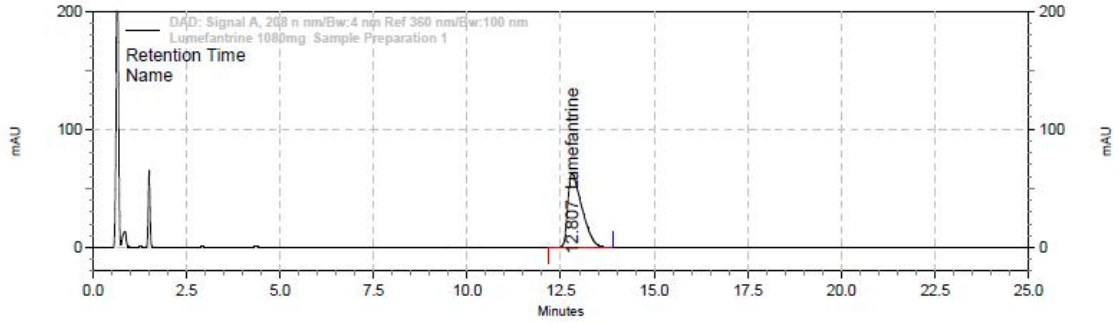
Change in the wave length - 208 nm – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.36	286806	9510	1.03

Chromatogram No – 7.69

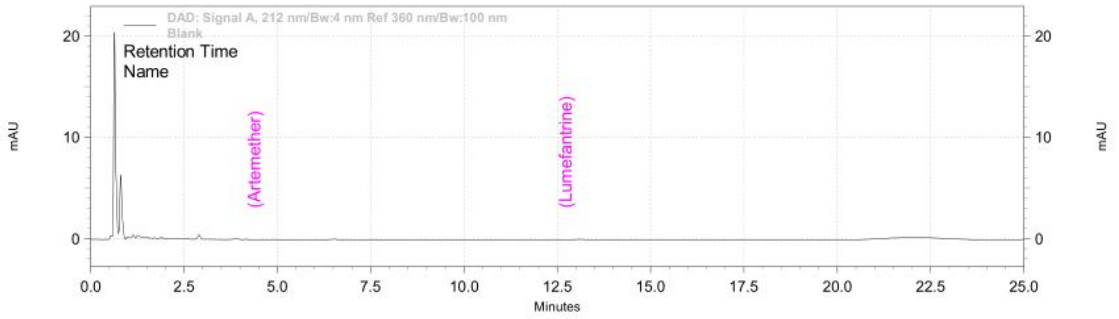
Change in the wave length - 208 nm – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.81	4464462	4944	1.92

Chromatogram No – 7.70

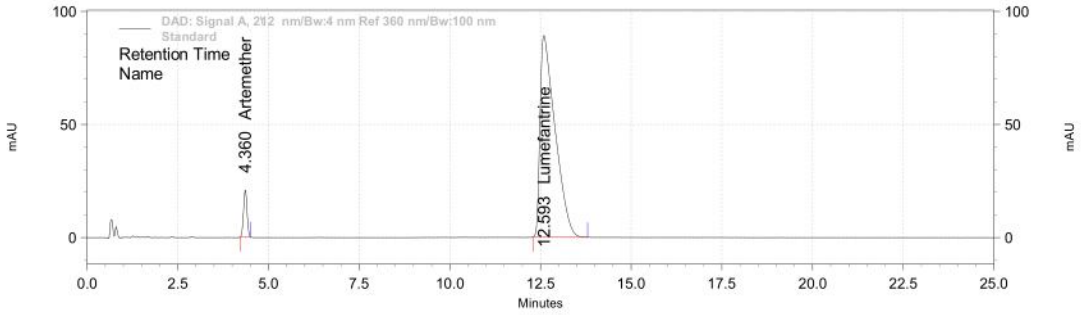
Change in the wave length - 212 nm - Blank



Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area

Chromatogram No – 7.71

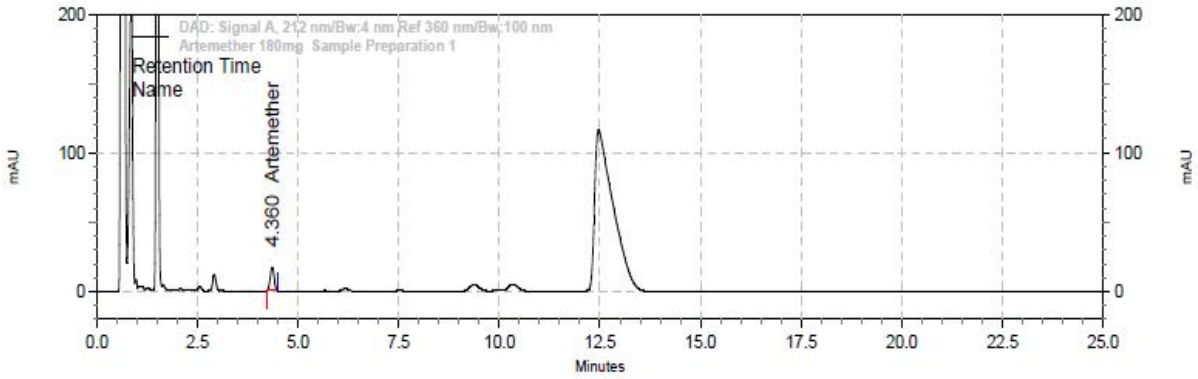
Change in the wave length – 212 nm – Standard



Peak Number	Name	Retention Time	Theoretical plates (USP)	Asymmetry	Resolution (USP)	Area
1	Artemether	4.36	9363	1.03	**0.00**	289143
2	Lumefantrine	12.59	3825	2.28	**16.58**	4972121

Chromatogram No – 7.72

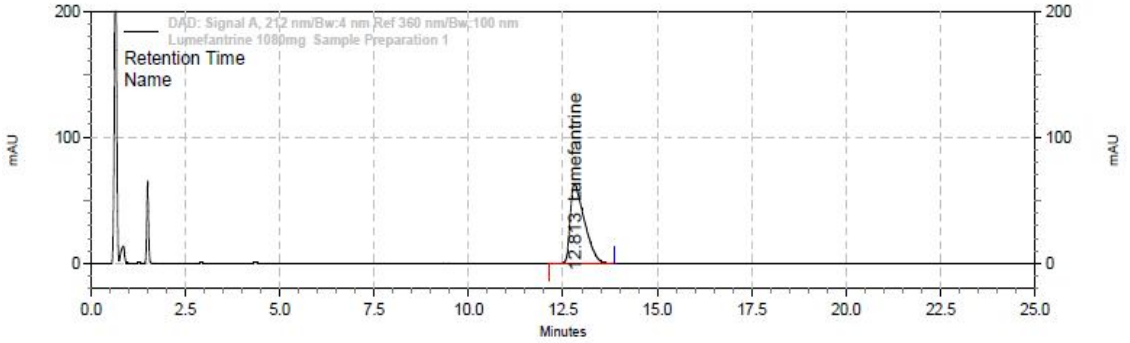
Change in the wave length – 212 nm – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.36	287186	9501	1.04

Chromatogram No – 7.73

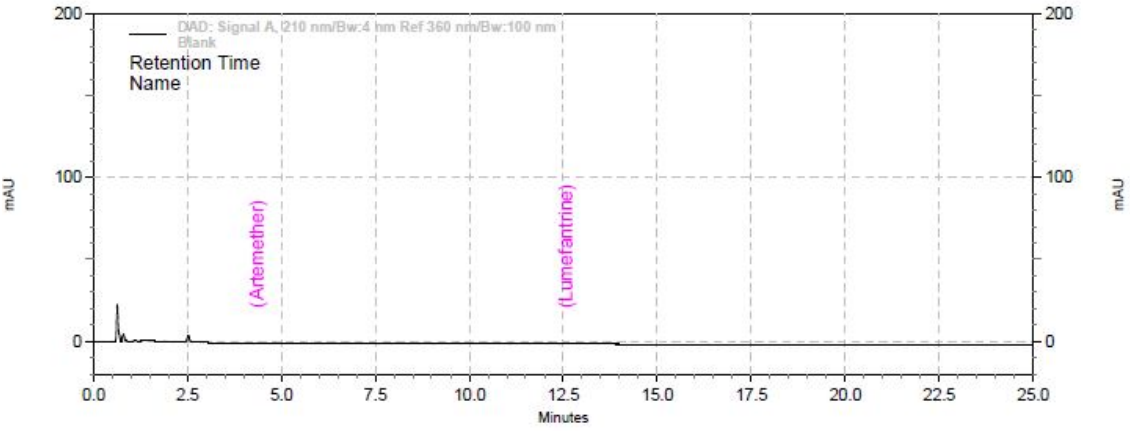
Change in the wave length -212 nm –Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.81	4472208	4945	1.89

Chromatogram No – 7.74

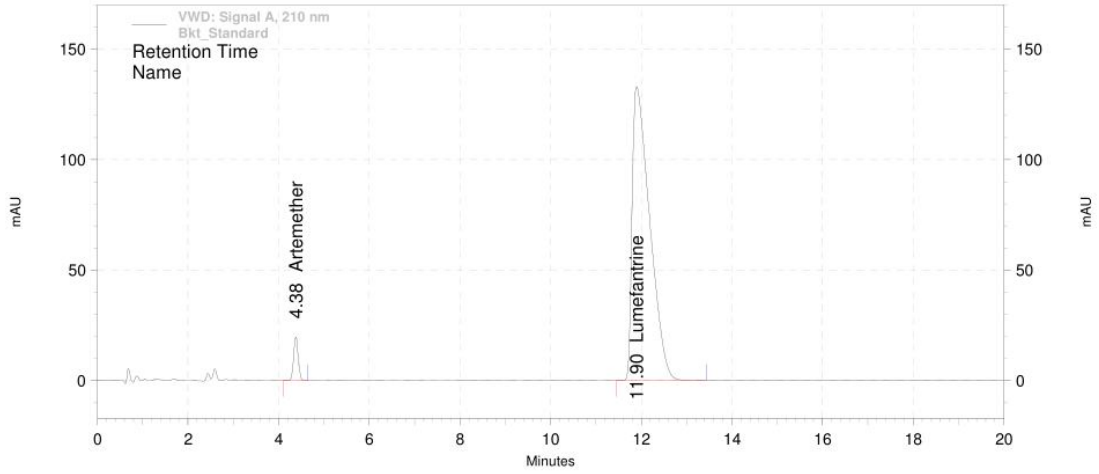
Change in the flow rate - 2.2 ml - Blank



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry

Chromatogram No – 7.75

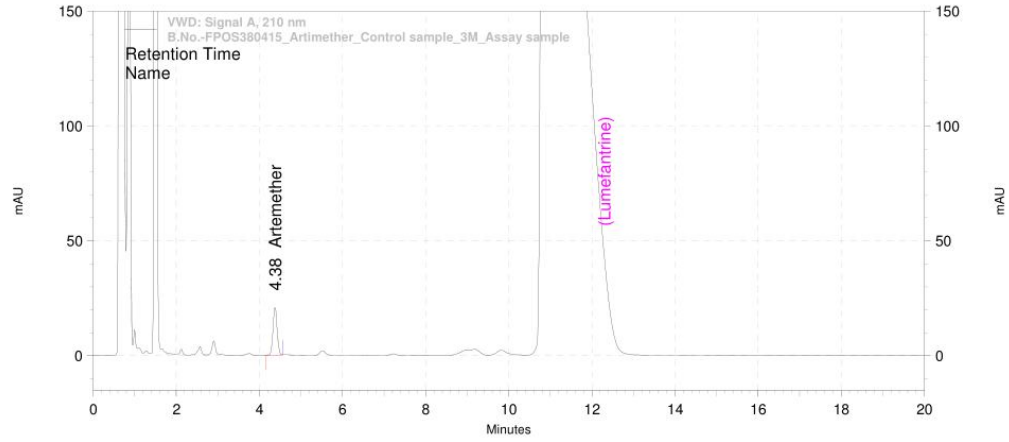
Change in the flow rate -2.2ml - Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.38	289416	9518	1.06	0.00
2	Lumefantrine	11.90	4977846	3608	2.33	16.52

Chromatogram No – 7.76

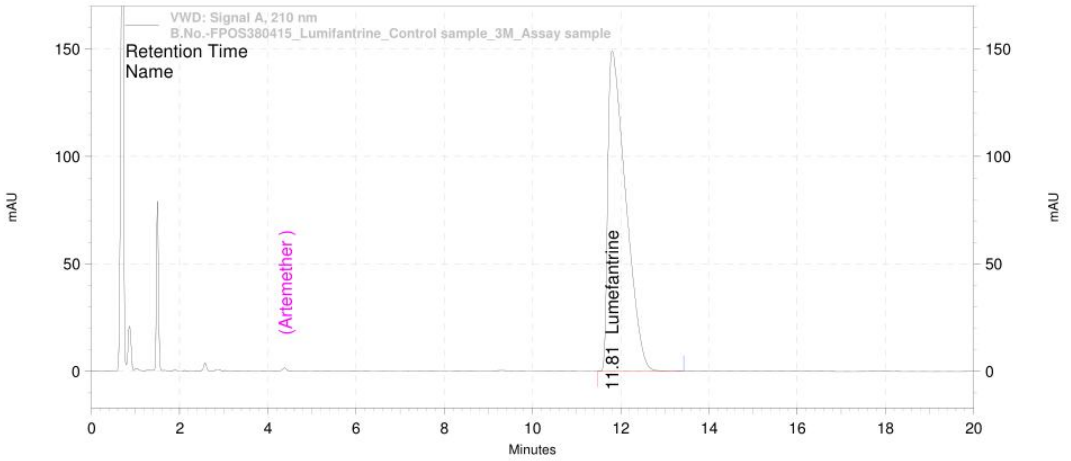
Change in the flow rate -2.2ml - Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.38	288636	9134	1.44	0.00

Chromatogram No – 7.77

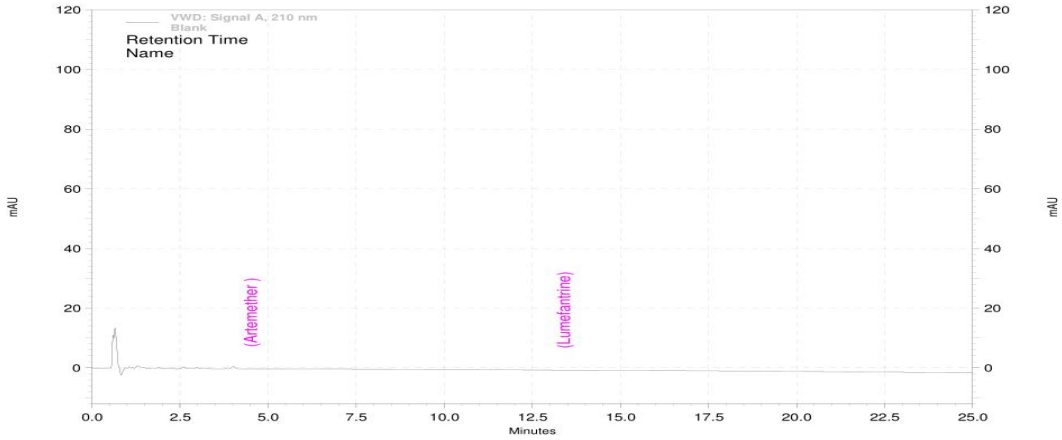
Change in the flow rate -2.2ml - Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Lumefantrine	11.81	4870449	3575	2.32	0.00

Chromatogram No – 7.78

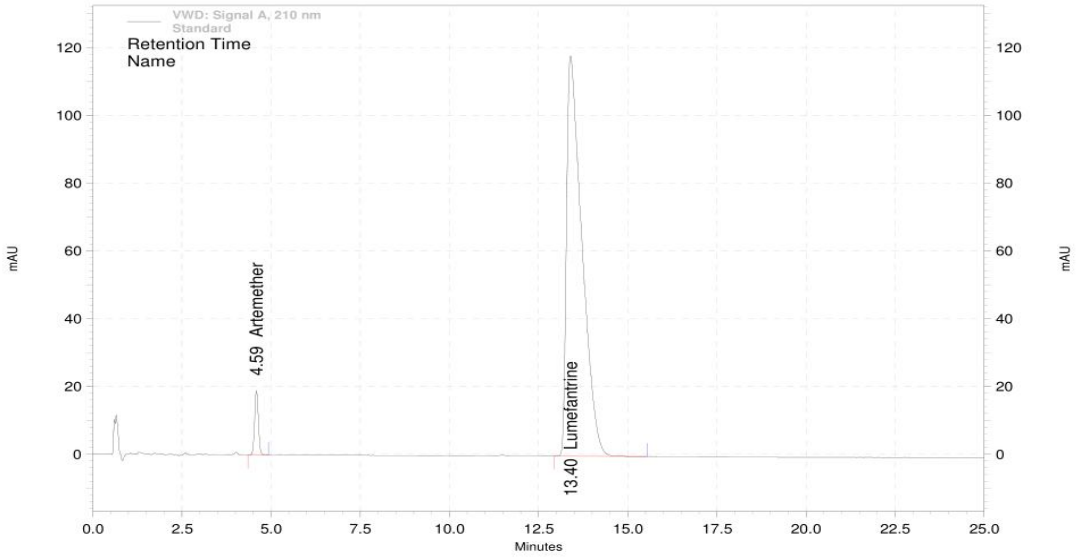
Change in the flow rate -1.8 ml – Blank



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	**Resolution (USP)**

Chromatogram No – 7.79

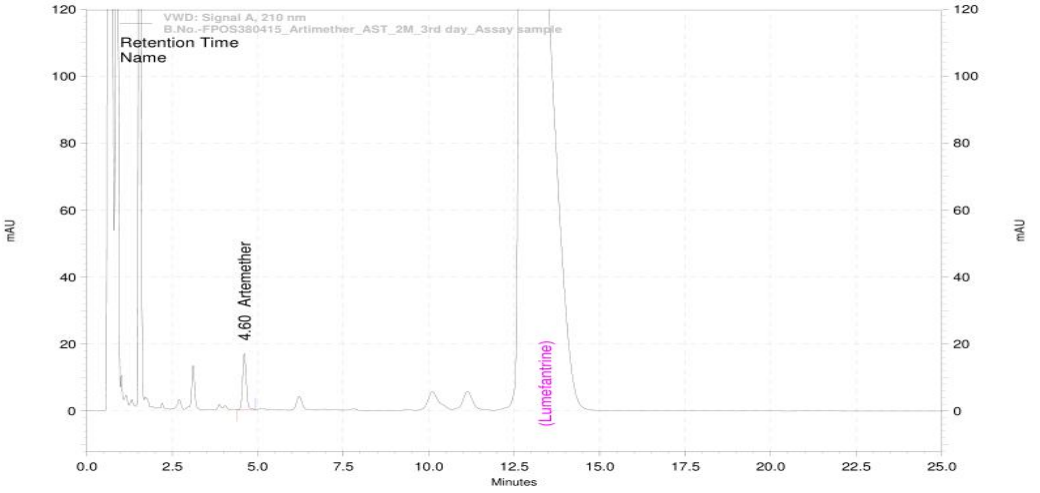
Change in the flow rate -1.8 ml - Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	**Resolution (USP)**
1	Artemether	4.587	282979	8923	1.05	**0.00**
2	Lumefantrine	13.397	4923140	4049	2.31	**17.00**

Chromatogram No – 7.80

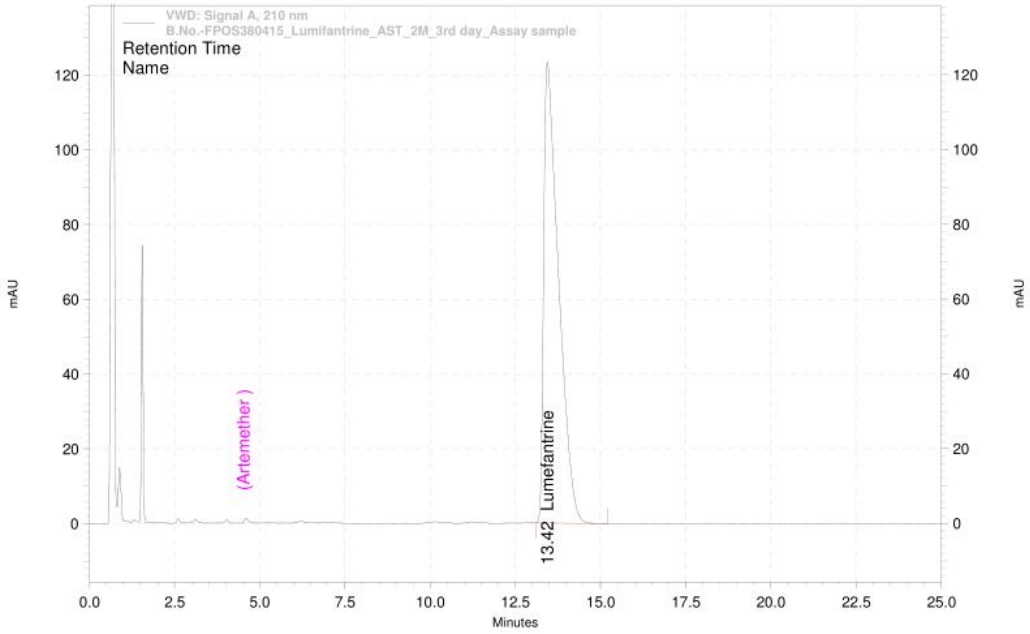
Change in the flow rate -1.8 ml - Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	**Resolution (USP)**
1	Artemether	4.597	284190	9226	1.09	**0.00**

Chromatogram No – 7.81

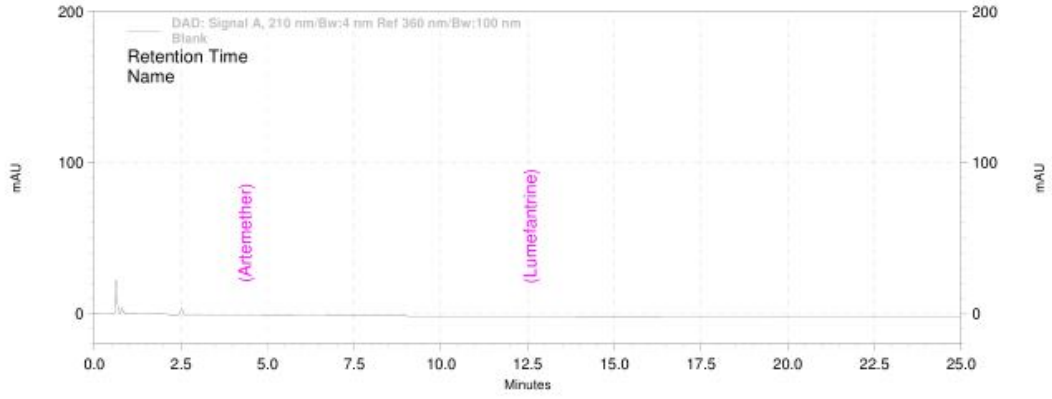
Change in the flow rate -1.8 ml - Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	**Resolution (USP)**
1	Lumefantrine	13.423	4760298	3940	2.41	**0.00**

Chromatogram No – 7.82

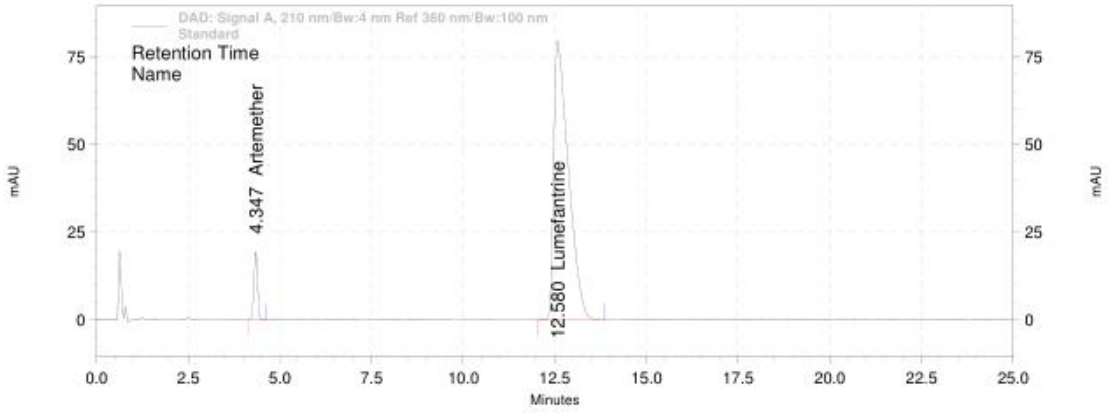
Change in mobile phase organic content plus 5% - Blank



Peak Number	**Name**	**Retention Time**	**Area**	**Theoretical plates (USP)**	**Asymmetry**	**Resolution (USP)**

Chromatogram No – 7.83

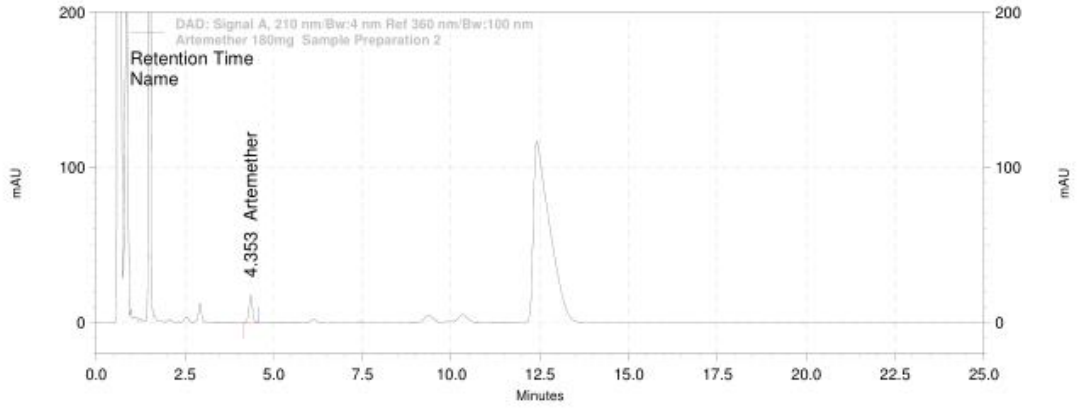
Change in mobile phase organic content plus 5% - Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	279046	9065	1.01	**0.00000**
2	Lumefantrine	12.58	4657708	4153	2.20	**17.09139**

Chromatogram No – 7.84

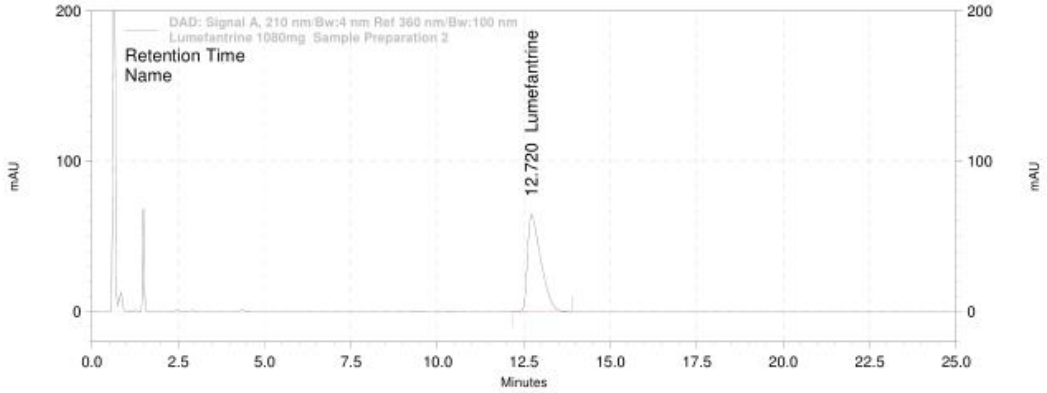
Change in mobile phase organic content plus 5% - Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	277150	9329	1.04

Chromatogram No – 7.85

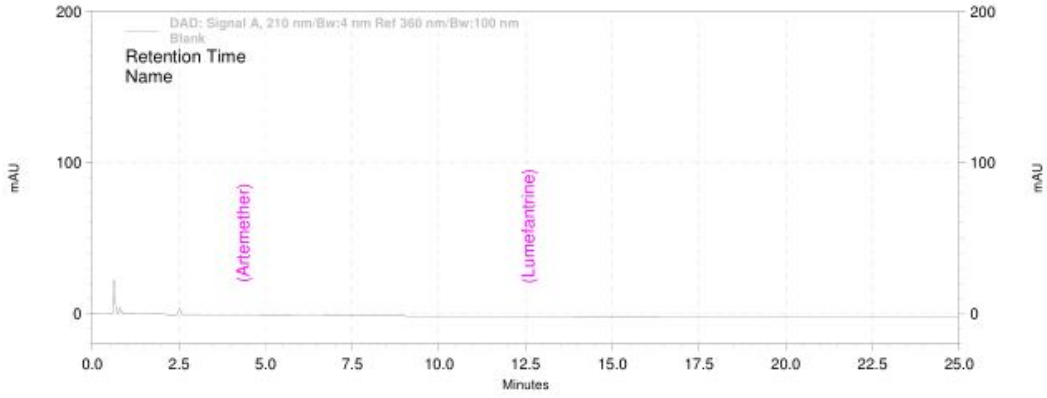
Change in mobile phase organic content plus 5% - Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.72	4291352	4798	1.97

Chromatogram No – 7.86

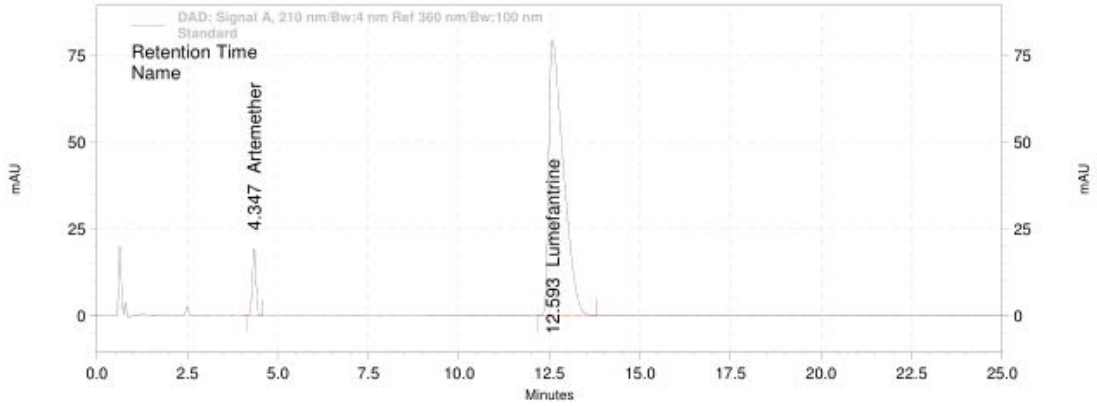
Change in mobile phase organic content minus 5% - Blank



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)

Chromatogram No – 7.87

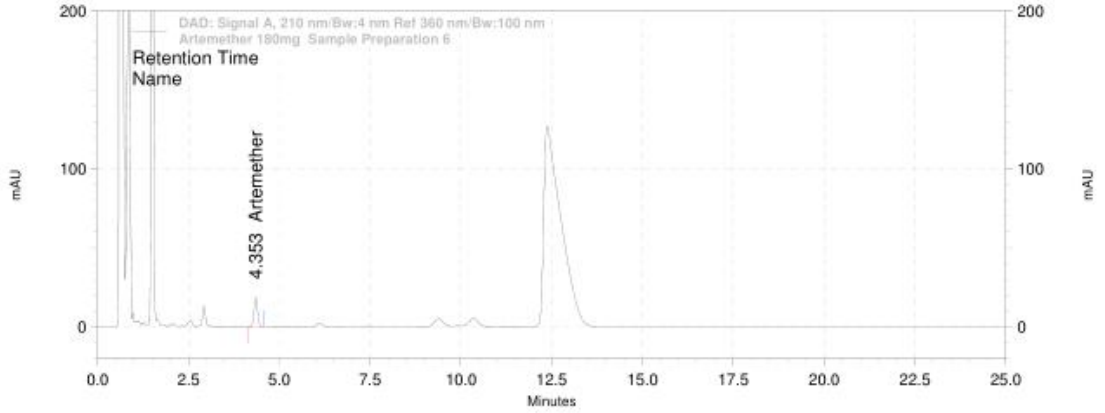
Change in mobile phase organic content minus 5% - Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	279362	9050	1.03	**0.00000**
2	Lumefantrine	12.59	4655473	4151	2.20	**17.09833**

Chromatogram No – 7.88

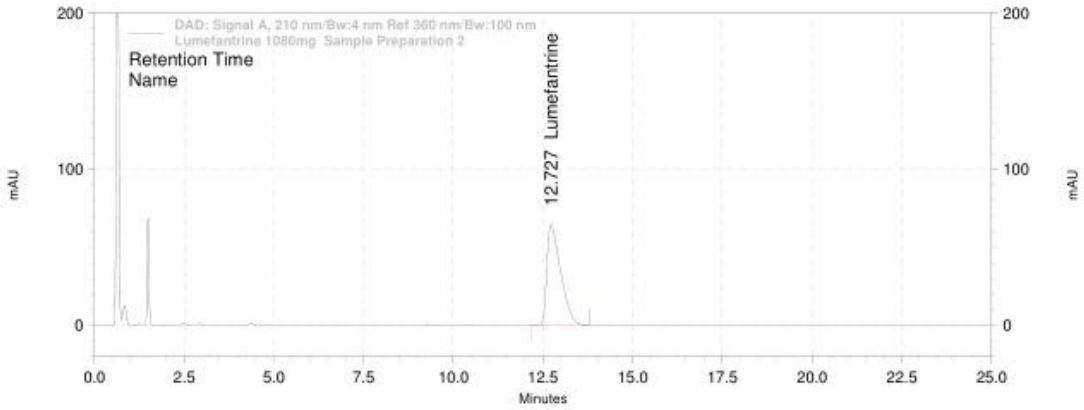
Change in mobile phase organic content minus 5% - Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	271712	9343	1.04

Chromatogram No – 7.89

Change in mobile phase organic content minus 5% - Sample Lumefantrine

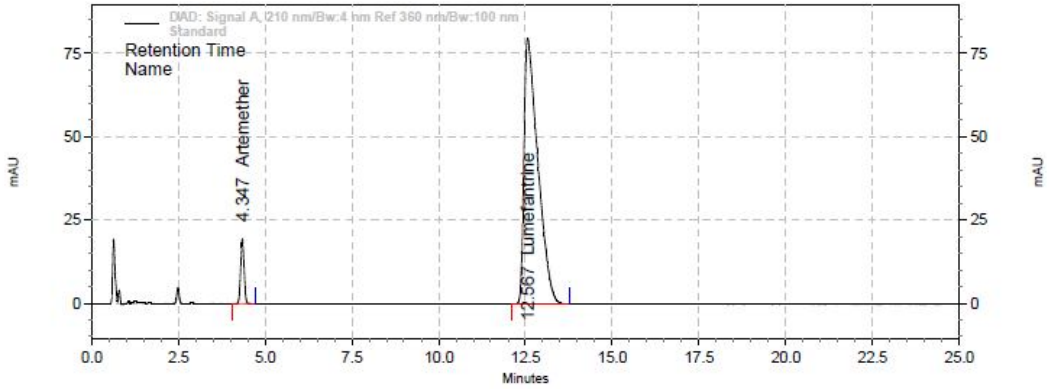


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.73	4292318	4799	1.96

STABILITY OF ANALYTICAL SOLUTIONS
(Standard, Sample)

Chromatogram No – 7.90

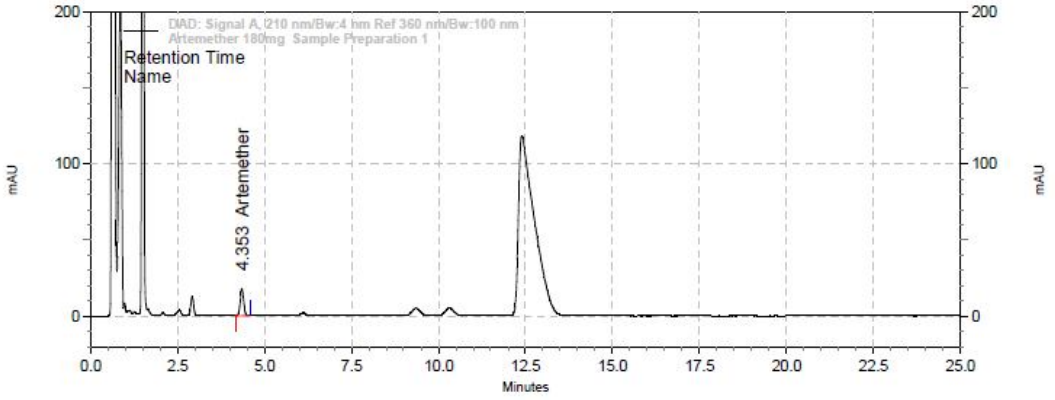
0 hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	289180	9382	1.01	**0.00000**
2	Lumefantrine	12.57	4972130	4165	2.19	**17.09351**

Chromatogram No – 7.91

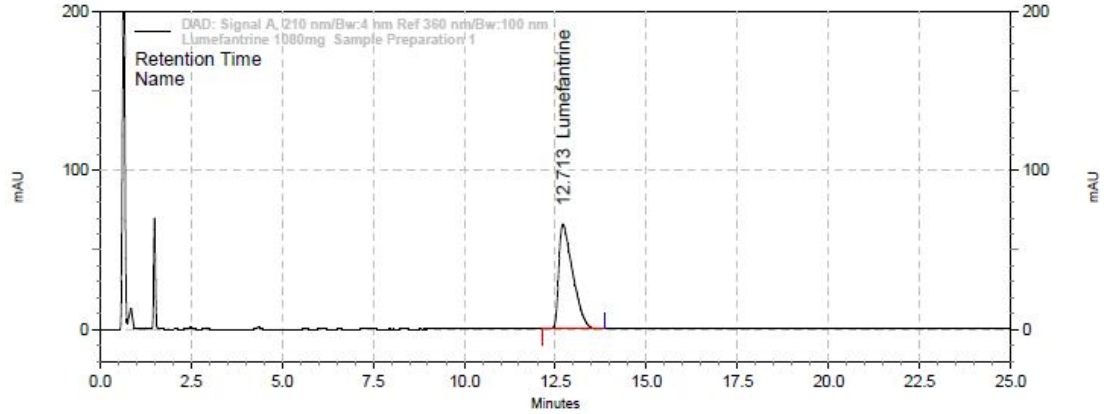
0 hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286899	9367	1.01

Chromatogram No – 7.92

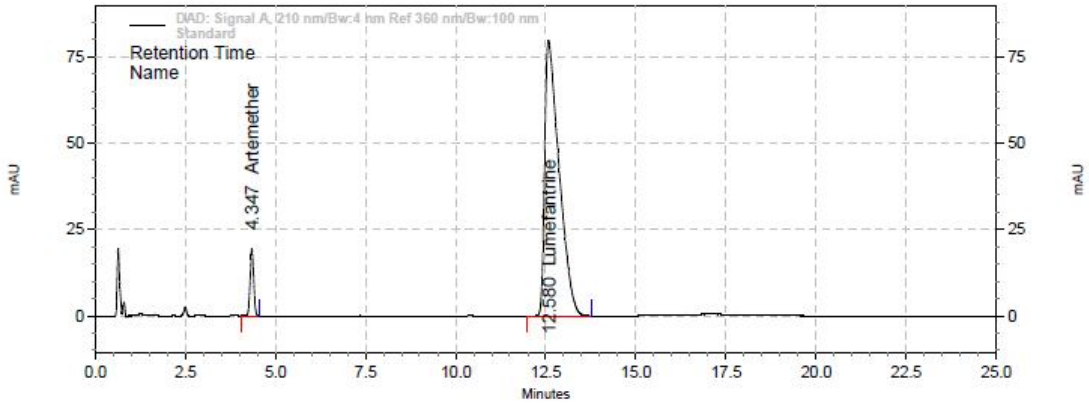
0 hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.71	4871372	4752	1.96

Chromatogram No – 7.93

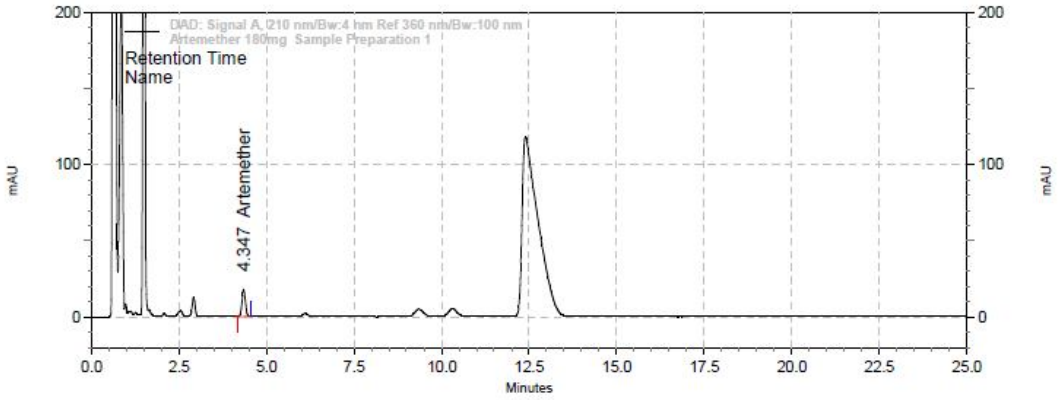
3rd hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	289290	9387	1.04	**0.00000**
2	Lumefantrine	12.58	4972651	4398	2.18	**17.09809**

Chromatogram No – 7.94

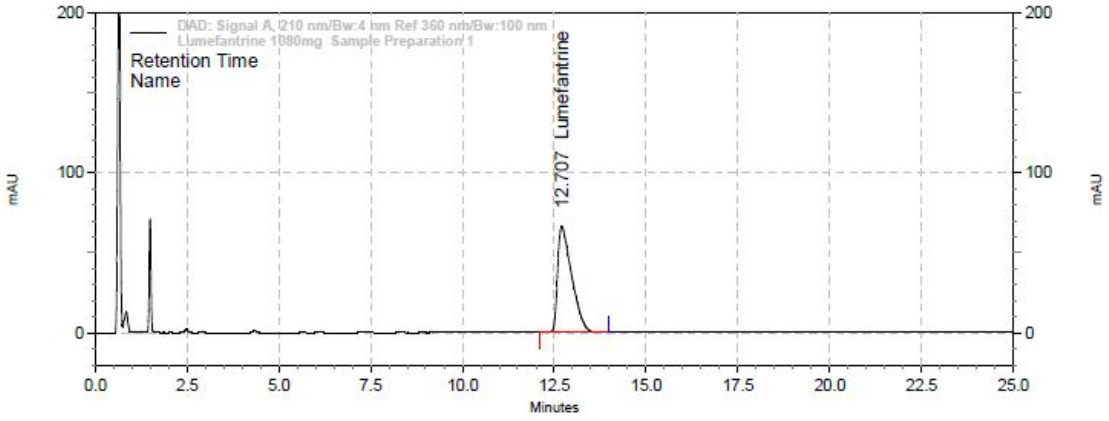
3rd hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286597	9339	1.06

Chromatogram No – 7.95

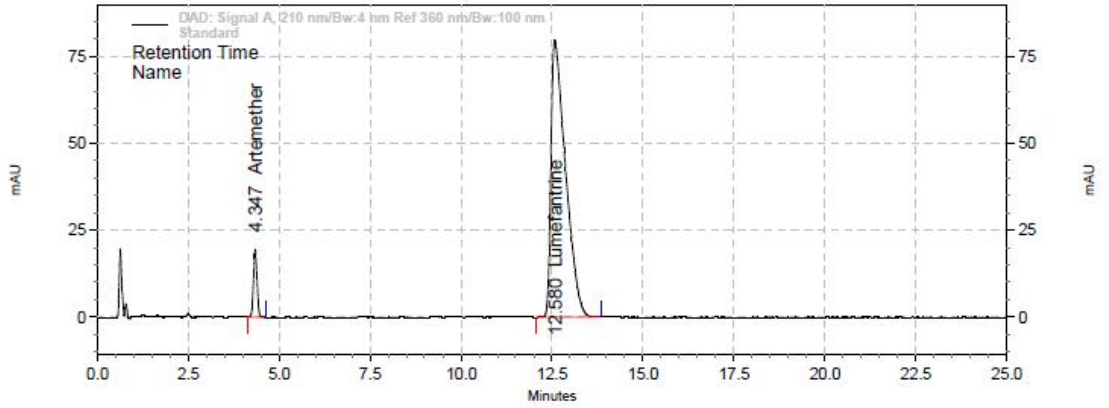
3rd hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.71	4892325	4723	1.99

Chromatogram No – 7.96

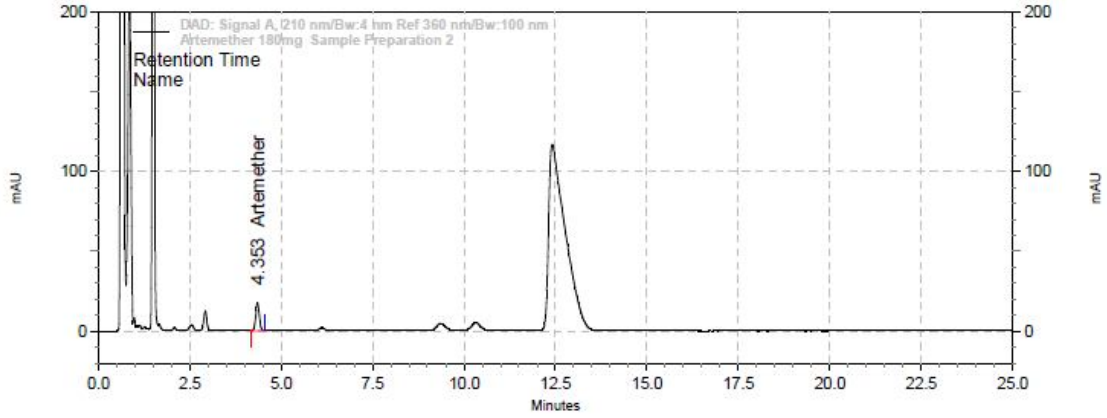
6th hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	289395	9401	1.01	**0.00000**
2	Lumefantrine	12.58	4973256	4157	2.20	**17.09139**

Chromatogram No – 7.97

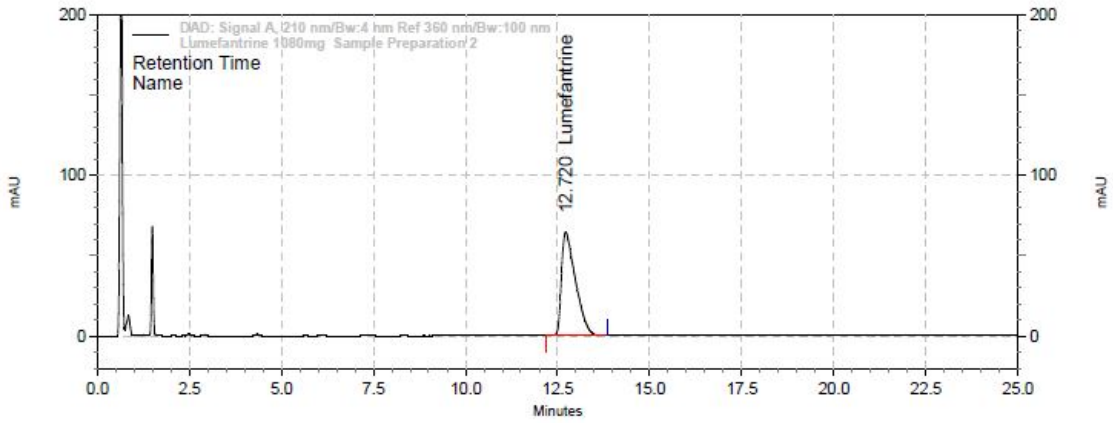
6th hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286456	9354	1.04

Chromatogram No – 7.98

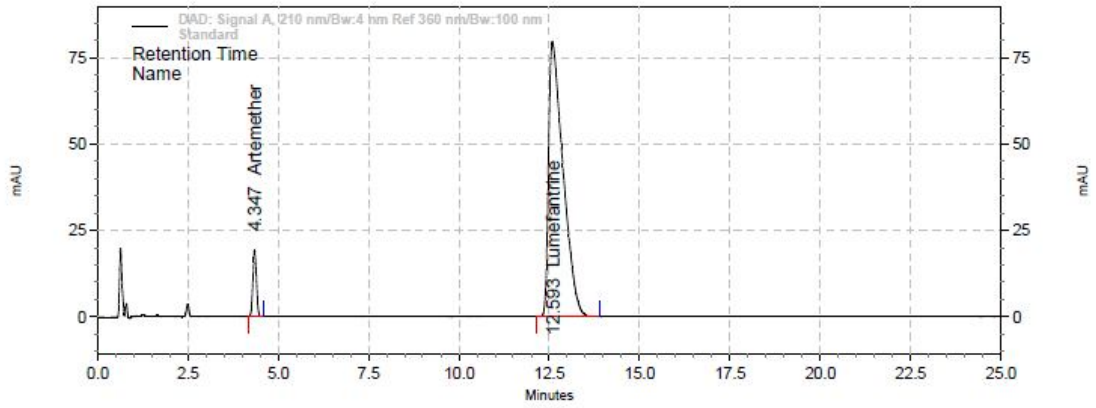
6th hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.72	4852837	4794	1.97

Chromatogram No – 7.99

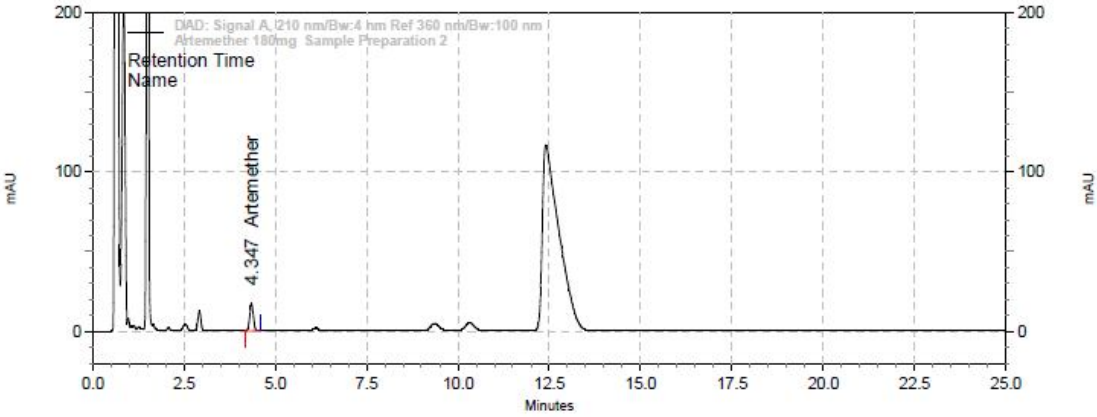
9th hour –Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	289178	9378	1.05	**0.00000**
2	Lumefantrine	12.59	4974785	4178	2.17	**17.11443**

Chromatogram No – 7.100

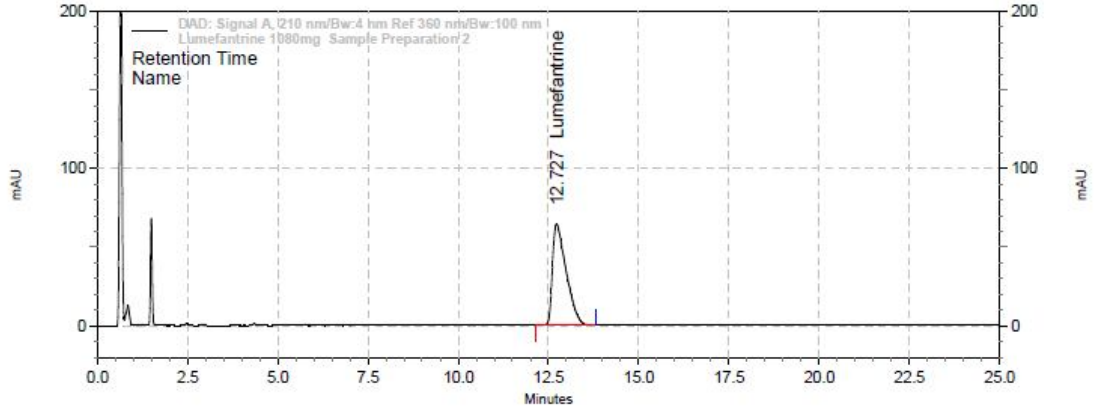
9th hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286954	9358	1.05

Chromatogram No – 7.101

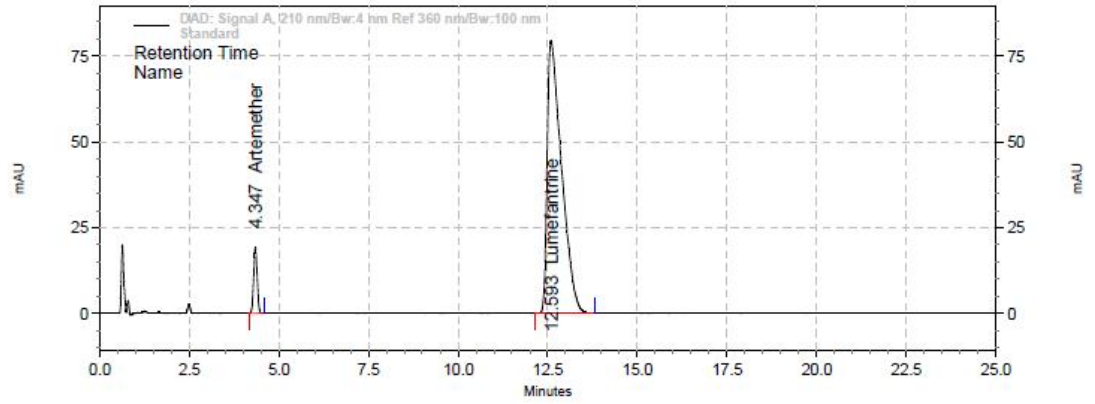
9th hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.73	4902014	4699	1.96

Chromatogram No – 7.102

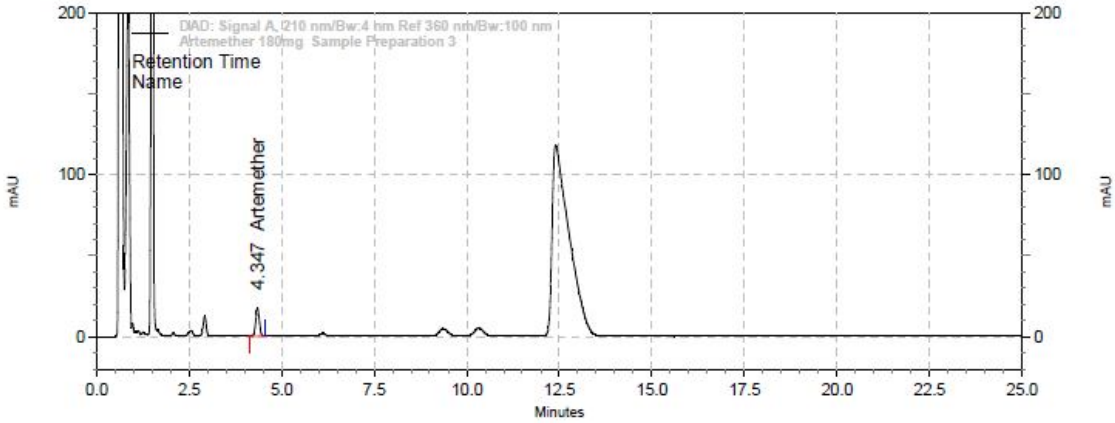
12th hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	288905	9409	1.03	**0.00000**
2	Lumefantrine	12.59	4972256	4165	2.20	**17.09833**

Chromatogram No – 7.103

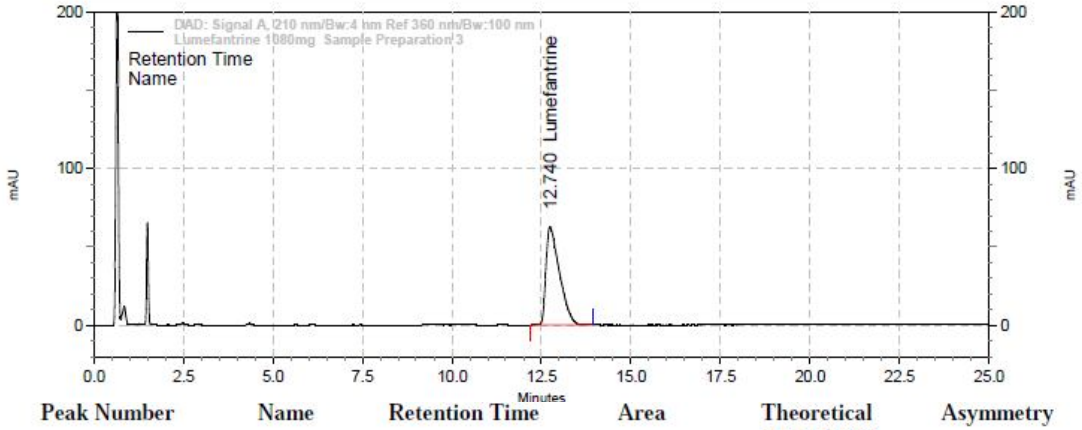
12th hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286546	9394	1.06

Chromatogram No – 7.104

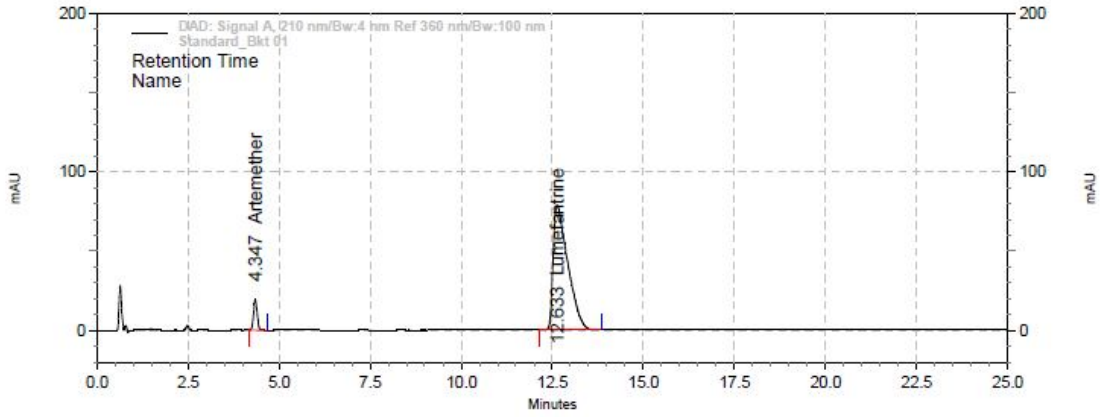
12th hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.74	4910045	4854	1.95

Chromatogram No – 7.105

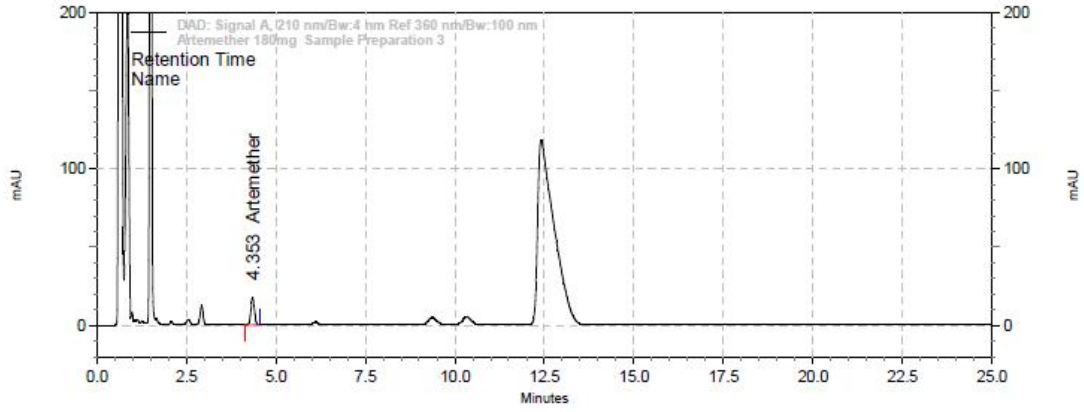
15th hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	289879	9415	1.05
2	Lumefantrine	12.63	4973125	4145	2.19

Chromatogram No – 7.106

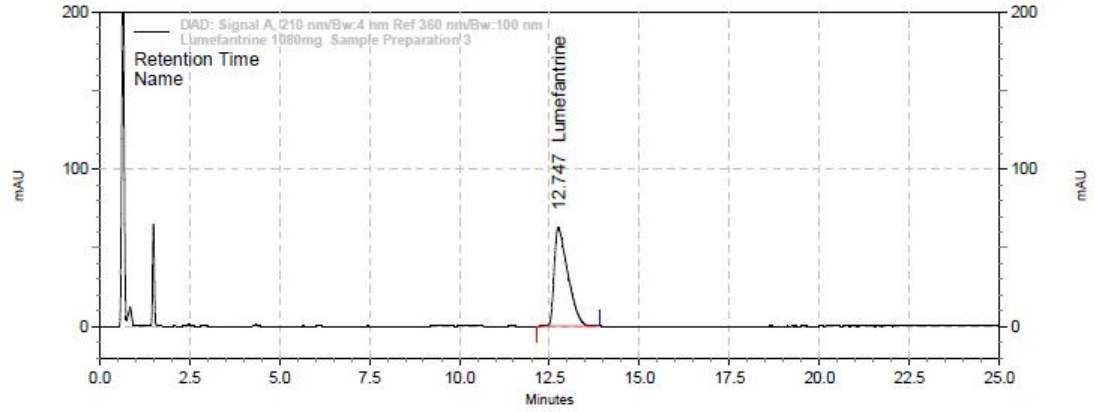
15th hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286785	9368	1.01

Chromatogram No – 7.107

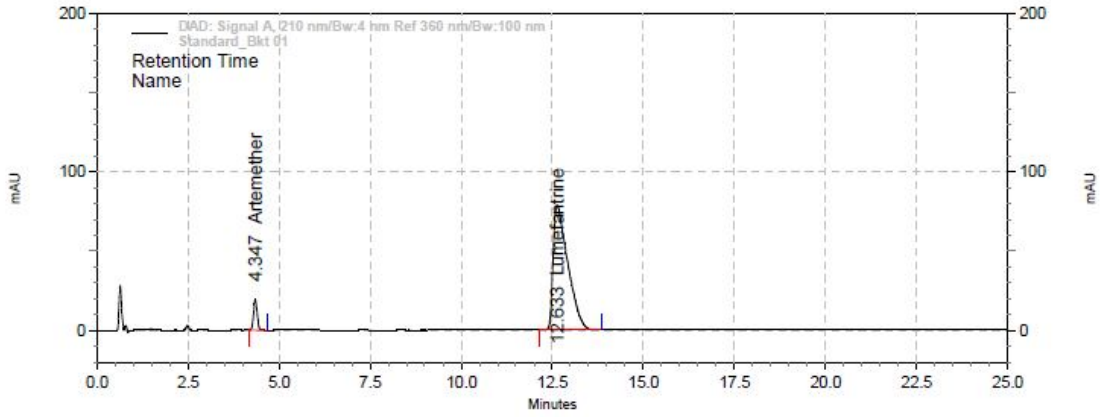
15th hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.75	4899074	4897	1.95

Chromatogram No – 7.108

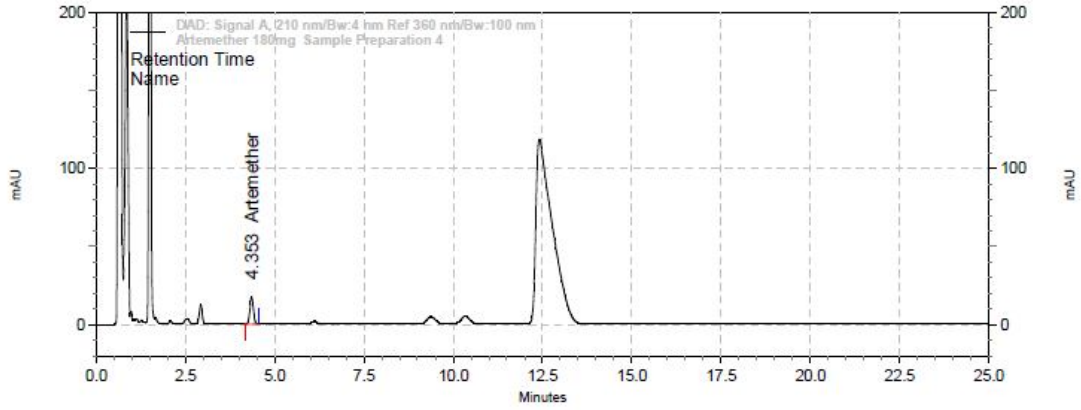
18th hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	289879	9415	1.05
2	Lumefantrine	12.63	4973125	4145	2.19

Chromatogram No – 7.109

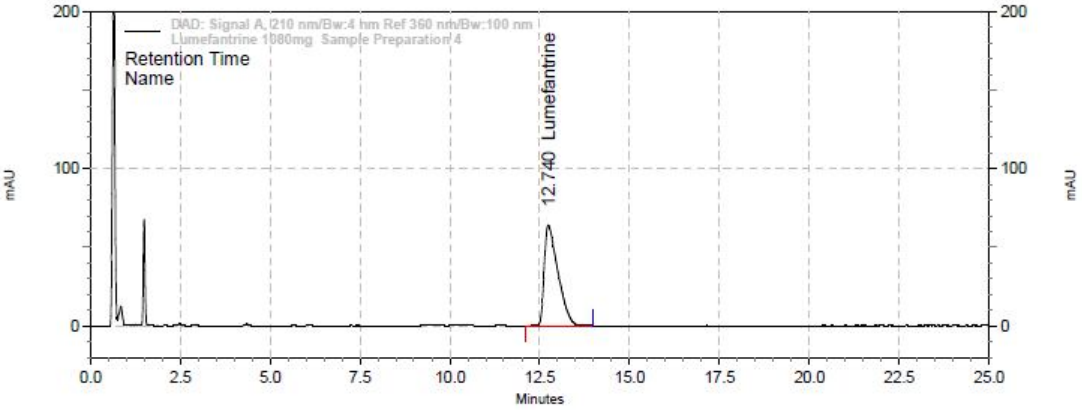
18th hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286546	9325	1.03

Chromatogram No – 7.110

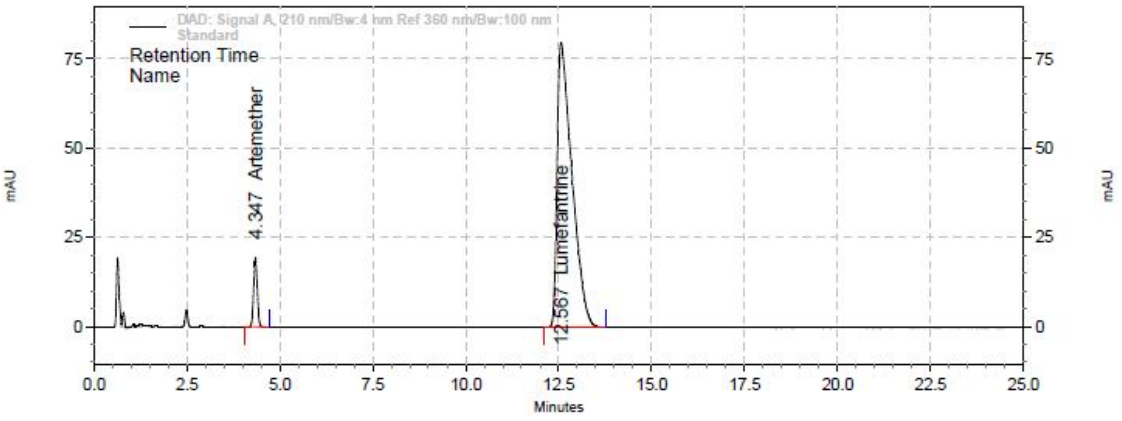
18th hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.74	4874021	4819	1.97

Chromatogram No – 7.111

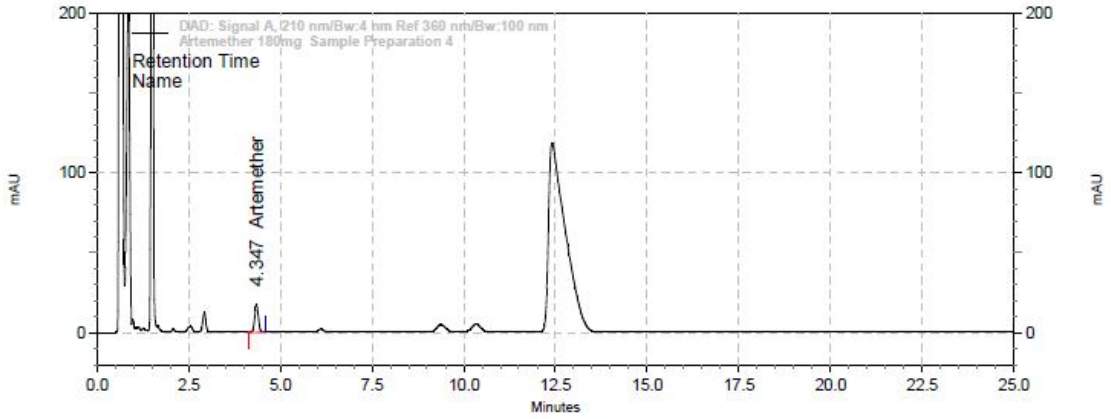
21st hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	288792	9358	1.01	**0.00000**
2	Lumefantrine	12.57	4964528	4018	2.19	**17.09351**

Chromatogram No – 7.112

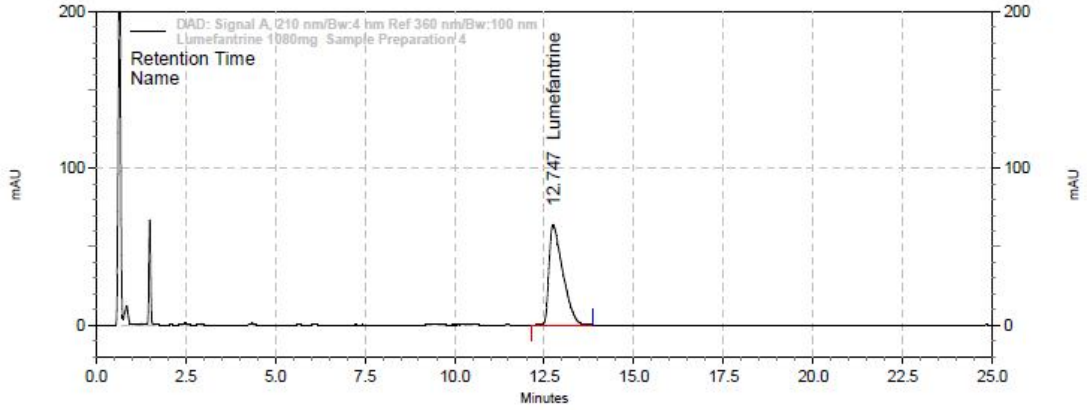
21st hour –Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286379	9255	1.06

Chromatogram No – 7.113

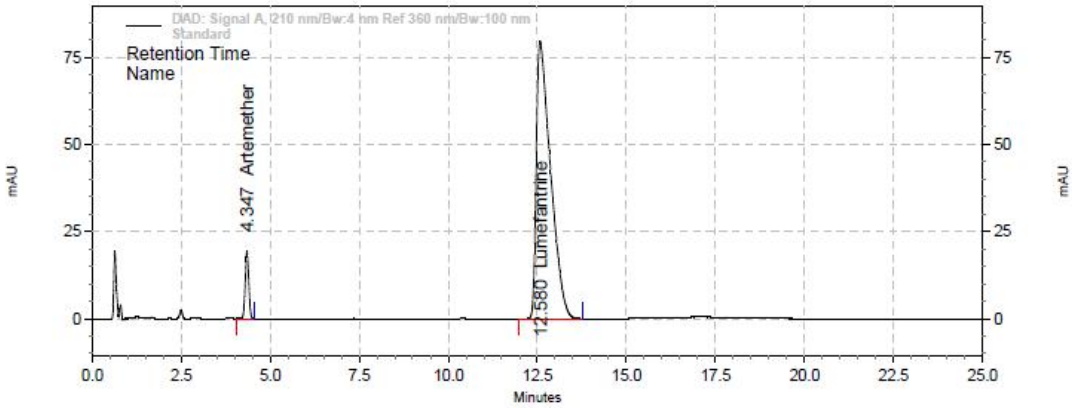
21st hour –Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.75	4872836	4845	1.99

Chromatogram No – 7.114

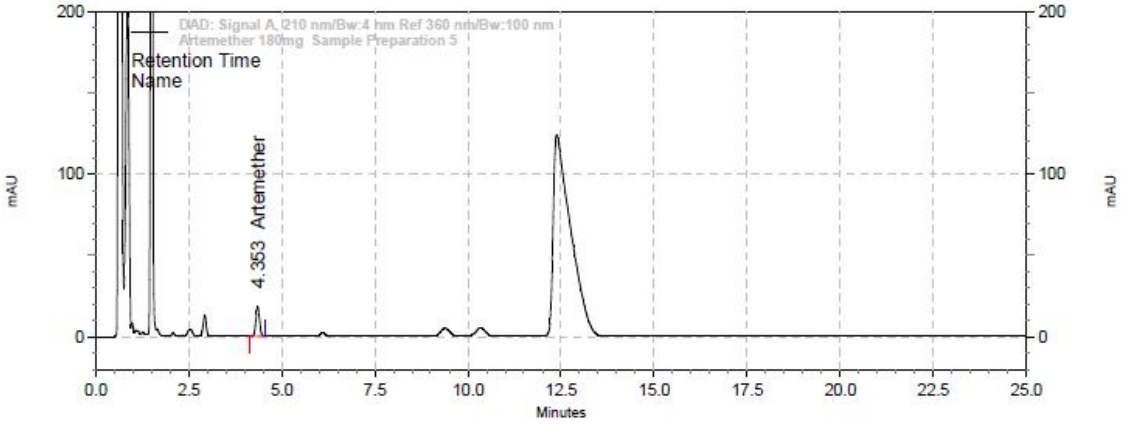
24th hour – Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry	Resolution (USP)
1	Artemether	4.35	289417	9417	1.04	**0.00000**
2	Lumefantrine	12.58	4977136	4187	2.18	**17.09809**

Chromatogram No – 7.115

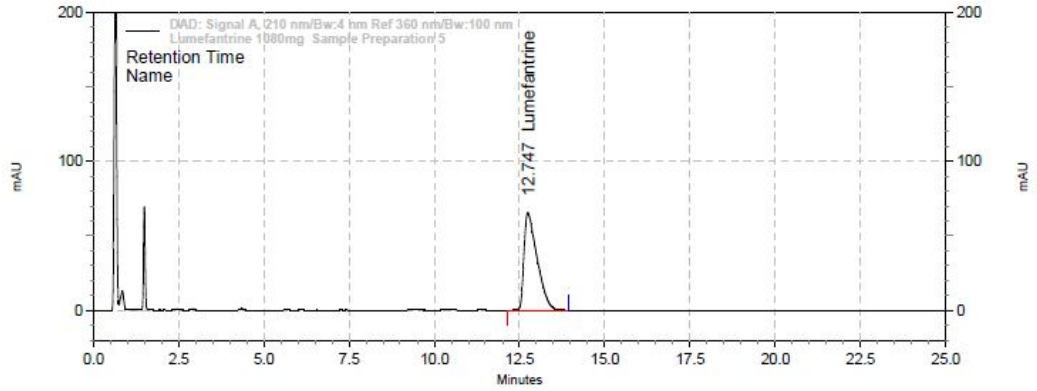
24th hour – Sample Artemether



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Artemether	4.35	286347	9378	1.03

Chromatogram No – 7.116

24th hour – Sample Lumefantrine



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Lumefantrine	12.75	4882235	4758	1.99

8. RESULT AND DISCUSSION

A simple Reverse phase high performance liquid chromatographic method has been developed and subsequently validated for Artemether and Lumefantrine powder for oral suspension dosage form (dry syrup).

The separation was carried out by using mobile phase composition selected for the chromatographic separation of Artemether and Lumefantrine was acetonitrile and phosphate buffer of pH 2.3 in the ration of 45:55 v/v. The detection was carried out at 210 nm. The column was Zorbax SB C_8 (150 x 4.6mm, 5µ). The flow rate was selected as 2mL/min.

SPECIFICITY

Table No: 8.1 Specificity System Suitability Report

S.No.	Parameter	Results obtained		Acceptance criteria
		Artemether	Lumefantrine	
01	Tailing factor from five replicate injections	1.03	2.32	**NMT 3.0**
02	Theoretical Plates from five replicate injections	9396	3843	**NLT 2000**
03	% RSD of area for five standard injections	0.100	0.089	**NMT 2.0 %**
04	Resolution between Artemether and Lumefantrine	17		**NLT 5.0**

Table No: 8.2 Specificity System Suitability Result

S. No.	Name of the solution	Results obtained	Acceptance criteria
01	Blank	No peak found in the retention time of principal peak	No any peak should be found in the retention time of Artemether and Lumefantrine.
02	Placebo	No peak found in the retention time of principalpeak	No any peak should be found in the retention time of Artemether and Lumefantrine.

Discussion for Specificity

System suitability result passes with reference to the table no. 8.1- 8.2 and the results obtained for specificity are found within the acceptance criteria. The obtained results proved that there will not be blank and placebo interference in the Artemether and Lumefantrine peak by this assay method. The chromatograms were showed in chromatogram no.7.4 to 7.8.

LINEARITY AND RANGE

Table No: 8.3 Linearity Report

S.No.	Parameter	Results obtained		Acceptance criteria
		Artemether	Lumefantrine	
01	Tailing factor from five replicate injections	1.03	2.28	**NMT 3.0**
02	Theoretical Plates from five replicate injections	9061	4096	**NLT 2000**
03	% RSD of area for five standard injections	0.030	0.027	**NMT 2.0 %**
04	Resolution between Artemether and Lumefantrine	16.79		**NLT 5.0**

Table No. 8.4 Linearity Data Obtained From 60 % to 160 % of Artemether

Linearity level concentration in %	Concentration of Artemether(mcg/mL)	Standard area -1	Standard area-2	Average area
60	192.0	179294	179233	179264
80	256.0	237715	237510	237613
100	320.0	290764	290592	290678
120	384.0	339031	339391	339211
160	512.0	455796	455684	455740

Figure 8.1 Linearity Graph Obtained for Artemether

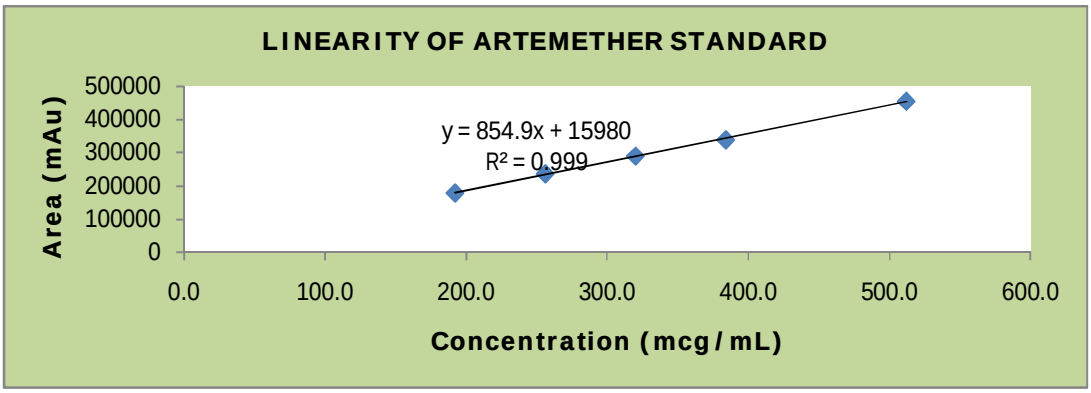


Table No. 8.5 Linearity Data Obtained From 60 % to 160 % of Lumefantrine

Linearity level concentration in %	Concentration of Lumefantrine (mcg/mL)	Standard area -1	Standard area-2	Average area
60	72.0	2979334	2977800	2978567
80	96.0	3983265	3979933	3981599
100	120.0	4876621	4886428	4881525
120	144.0	5662400	5663851	5663126
160	192.0	7694804	7724812	7709808

Figure 8.2 Linearity Graph Obtained for Lumefantrine

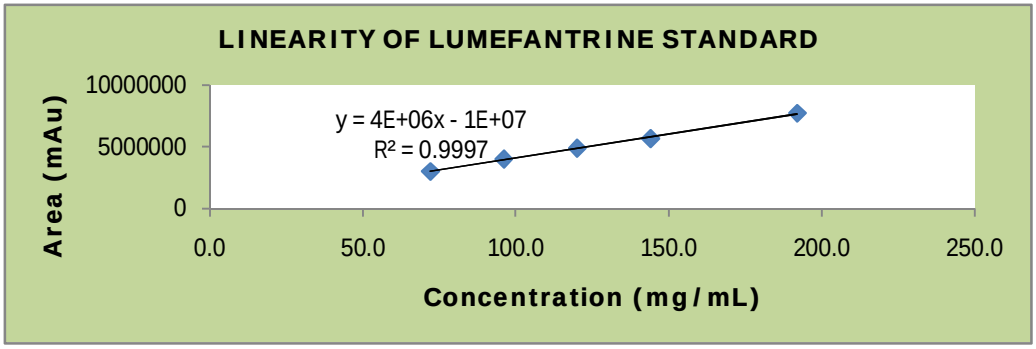


Table No: 8.6 Linearity Results

S.No.	Parameter	Results obtained		Acceptance criteria
		Artemether	Lumefantrine	
01	Correlation coefficient	0.99956	0.99911	**NLT 0.998**
02	Regression coefficient	0.99911	0.9982	**Informative**
03	Residual sum of squares	Not Applicable		**Informative**
04	Intercept	15980.01351	190449.3378	**Informative**
05	Slope of regression line	854.9308488	38882.01492	**Informative**

Discussion for Linearity

System suitability result passes and the results obtained for linearity are found within the acceptance criteria with reference to the table 8.3 to 8.6. Hence it is concluded that the range of concentrations, 60 % to 160 % with respect to 100 %

working concentration for assay method is linear for Artemether and Lumefantrine. The chromatograms were showed in chromatogram no.7.9 to 7.15.

PRECISION

System Precision

Table No: 8.7 System Precision Reports for System Suitability

S.No.	Parameter	Results obtained		Acceptance criteria
		Artemether	Lumefantrine	
01	Tailing factor from five replicate injections	1.03	2.32	**NMT 3.0**
02	Theoretical Plates from five replicate injections	9096	4162	**NLT 2000**
03	% RSD of area for five standard injections	0.100	0.089	**NMT 2.0 %**
04	% RSD of retention time for five standard injections	1.03	2.32	**NMT 1.0 %**
05	Resolution between Artemether and Lumefantrine	17		**NLT 5.0**

Table No: 8.8 System Precision Results for Artemether

S. No.	Retention	Standard Area	Tailing factor	Theoretical plates
01	4.36	289180	1.04	9380
02	4.36	289578	1.05	9390
03	4.36	288814	1.02	9391
04	4.36	289411	1.02	9402
05	4.36	289339	1.01	9418
Mean	**4.36**	**289264**	**1.03**	**9396**
Std dev	**0.000**	**289.56**	**NMT 3.0**	**NLT 2000**
(RSD %)	**0.00**	**0.100**		
Limit	**NMT 1.0 %**	**NMT 2.0 %**		

Table No: 8.9 System precision Result for Lumefantrine

S. No.	Retention	Standard Area	Tailing factor	Theoretical
01	12.59	4972130	2.33	3836
02	12.61	4911680	2.31	3854
03	12.61	4913540	2.29	3848
04	12.61	4927650	2.35	3841
05	12.61	4932990	2.30	3834
Mean	**12.61**	**4931598.00**	**2.32**	**3843**
Stddev	**0.009**	24408.94	**NMT 3.0**	**NLT 2000**
(RSD %)	**0.07**	0.49		
Limit	**NMT 1.0 %**	**NMT 2.0 %**		

Discussion for system precision:

All the system suitability parameters were well within the desirable limits with reference to the table no.8.7 to 8.9, it indicates that the prescribed method is suitable to perform the estimation of Artemether and Lumefantrine from of Artemether and Lumefantrine for Oral Suspension (180 mg & 1080 mg).Further there was no deviation in the given method. The chromatograms were showed in the chromatogram no.7.16 to 7.17.

Method Precision (Repeatability)

Table No: 8.10 System Suitability Result in Standard solution

S.No.	Parameter	Results obtained		Acceptance criteria
		Artemether	Lumefantrine	
01	Tailing factor from five replicate injections	1.03	2.32	**NMT 3.0**
02	Theoretical Plates from five replicate injections	9396	3843	**NLT 2000**
03	% RSD of area for five standard injections	0.100	0.089	**NMT 2.0 %**
04	Resolution between Artemether and Lumefantrine	17		**NLT 5.0**

Table No: 8.11 Method Precision Results for Artemether

No. of Sample preparation	Samples Area			Results obtained		Acceptance criteria
	Sample -1	Sample -2	Average	Amount of drug present in mg	Percentage of drug	
01	286806	287186	**286996**	**180.43**	**100.24**	**90.0% - 110.0%**
02	285326	285340	**285333**	**178.77**	**99.32**	
03	286393	286196	**286295**	**176.98**	**98.32**	
04	285938	285770	**285854**	**181.56**	**100.87**	
05	287828	287882	**287855**	**183.46**	**101.92**	
06	289119	288834	**288977**	**180.45**	**100.25**	
				Mean	**100.15**	
				Std. Dev	**1.242**	
				RSD	**1.240**	**NMT 2.0 %**

Table No.8.12 Method Precision results for Lumefantrine

No. of Sample preparation	Samples Area			Results obtained		Acceptance criteria
	Sample -1	Sample -2	Average	Amount of drug present in mg	Percentage of drug	
01	4464462	4472208	**4468335**	**1087.86**	**100.73**	**90.0% - 110.0%**
02	4456902	4462076	**4459489**	**1073.71**	**99.42**	
03	4429942	4429292	**4429617**	**1066.51**	**98.75**	
04	4456342	4458593	**4457468**	**1079.18**	**99.92**	
05	4489968	4495105	**4492537**	**1081.66**	**100.15**	
06	4441994	4443336	**4442665**	**1075.60**	**99.59**	
				Mean	**1077.42**	
				Std. dev	**7.296**	
				RSD	**0.677**	**NMT 2.0 %**

Discussion for Method precision:

System suitability parameters were well within the prescribed limits with reference to the table no.8.10 to 8.12, which revealed that the prescribed procedure is capable to perform method precision using sample preparation. All the performed samples showed results between 90.0 % and 110.0 %.The method precision parameter complies as per In-House specification. The chromatograms were showed in chromatogram no.7.18 to 7.31.

ACCURACY

Table No: 8.13 System Suitability Results for Accuracy

S.No.	Parameter	Results obtained		Acceptance criteria
		Artemether	Lumefantrine	
01	Tailing factor from five replicate injections	1.03	2.28	**NMT 3.0**
02	Theoretical Plates from five replicate injections	9061	4096	**NLT 2000**
03	% RSD of area for five standard injections	0.030	0.027	**NMT 2.0 %**
04	Resolution between Artemether and Lumefantrine	16.76		**NLT 5.0**

Table No: 8.14 Accuracy Results for Artemether

Accuracy Level in %	Artemether added in mg	Artemether recovered in mg	% Recovered	Mean of % Recovered	Acceptance criteria
50	0.159851	0.160055	100.13	**100.01%**	**98.0 % - 102.0 %**
	0.161044	0.160729	99.80		
	0.160249	0.160407	100.10		
100	0.318112	0.324047	101.87	**100.67%**	
	0.322884	0.326964	101.26		
	0.318112	0.314598	98.90		
150	0.478759	0.481611	100.60	**100.55%**	
	0.487109	0.495009	101.62		
	0.481940	0.479169	99.43		

Table No: 8.15 Accuracy Results for Artemether

Accuracy Level in %	Artemether added in mg	Artemether recovered in mg	% Recovered	Mean of % Recovered	Acceptance criteria
50	0.159851	0.160055	100.13	**100.01%**	**98.0 % - 102.0 %**
	0.161044	0.160729	99.80		
	0.160249	0.160407	100.10		
100	0.318112	0.324047	101.87	**100.67%**	
	0.322884	0.326964	101.26		
	0.318112	0.314598	98.90		
150	0.478759	0.481611	100.60	**100.55%**	
	0.487109	0.495009	101.62		
	0.481940	0.479169	99.43		

Table No.8.16 Accuracy Results for Lumefantrine

Accuracy Level in %	Lumefantrine added in mg	Lumefantrine recovered in mg	% Recovered	Mean of % Recovered	Acceptance criteria
50	0.063263	0.064060	101.26	**100.66%**	**98.0 % - 102.0 %**
	0.064457	0.064684	100.35		
	0.064059	0.064298	100.37		
100	0.124139	0.125100	100.77	**100.66%**	
	0.121353	0.122150	100.66		
	0.122547	0.123206	100.54		
150	0.187004	0.190144	101.68	**101.59%**	
	0.183821	0.186764	101.60		
	0.191778	0.194656	101.50		

Discussion for Accuracy:

System suitability result passes and the results obtained for accuracy are found within the acceptance criteria with reference to the table no.8.13 to 8.16. The chromatograms were showed in chromatogram no. 7.32 to 7.51.

RUGGEDNESS (INTERMEDIATE PRECISION)

Table No: 8.17 System Suitability Results for Ruggedness

S.No.	Parameter	Results obtained		Acceptance criteria
		Artemether	Lumefantrine	
01	Tailing factor from five replicate injections	1.03	2.19	**NMT 3.0**
02	Theoretical Plates from five replicate injections	9051	4158	**NLT 2000**
03	% RSD of area for five standard injections	0.227	0.047	**NMT 2.0 %**
04	Resolution between Artemether and Lumefantrine	17.1		**NLT 5.0**

Table No: 8.18 Intermediate Precision Results for Artemether

No. of Sample preparation	Samples Area			Results obtained		Acceptance criteria
	Sample - 1	Sample -2	Average	Amount of drug present in mg	Percentage of drug	
1	273295	273115	**273205**	**177.64**	**98.69**	**90.0 to 110.0 %**
2	277150	277029	**277090**	**178.34**	**99.08**	
3	278416	278465	**278441**	**176.83**	**98.24**	
4	278493	278474	**278484**	**179.85**	**99.92**	
5	270769	270380	**270575**	**178.97**	**99.43**	
6	271712	271635	**271674**	**176.64**	**98.13**	
				Average	**98.91**	
				Std. Dev	**0.695**	
				RSD	**0.702**	**NMT 2.0 %**

Table No: 8.19 Intermediate Precision Results for Lumefantrine

No. of Sample preparation	Samples Area			Results obtained		Acceptance criteria
	Sample -1	Sample -2	Average	Amount of drug present in mg	Percentage of drug	
1	4289102	4218029	**4253566**	**1067.26**	**98.82**	**90.0 % - 110.0 %**
2	4291352	4292318	**4291835**	**1094.72**	**101.36**	
3	4258195	4261537	**4259866**	**1104.87**	**102.30**	
4	4260643	4262731	**4261687**	**1075.15**	**99.55**	
5	4261898	4268101	**4265000**	**1070.13**	**99.09**	
6	4280265	4290892	**4285579**	**1093.12**	**101.21**	
				Average	**100.39**	
				Std. Dev	**1.425**	
				RSD	**1.420**	**NMT2%**

Table No: 8.20 Combined Method Precision and Intermediate Precision Results for Artemether

Sample preparation	Results obtained		Acceptance criteria
	Artemether drug release in mg	Artemether drug release in %	
Method Precision-1	180.43	100.24	**90.0% - 110.0%**
Method Precision-2	178.77	99.32	
Method Precision-3	176.98	98.32	
Method Precision-4	181.56	100.87	
Method Precision-5	183.46	101.92	
Method Precision-6	180.45	100.25	
Intermediate precision-1	177.64	98.69	
Intermediate precision-2	178.34	99.08	
Intermediate precision-3	176.83	98.24	
Intermediate precision-4	179.85	99.92	
Intermediate precision-5	178.97	99.43	
Intermediate precision-6	176.64	98.13	
Mean		**99.53**	
Std Dev		**1.16**	
% RSD		**1.16**	**NMT 2.0 %**

Table No. 8.21 Combined Method Precision and Intermediate Precision Results for Lumefantrine

Sample preparation	Results obtained		Acceptance criteria
	Lumefantrine drug release in mg	Lumefantrine drug release in %	
Method Precision-1	1087.86	100.73	**90.0% - 110.0%**
Method Precision-2	1073.71	99.42	
Method Precision-3	1066.51	98.75	
Method Precision-4	1079.18	99.92	
Method Precision-5	1081.66	100.15	
Method Precision-6	1075.60	99.59	
Intermediate precision-1	1067.26	98.82	
Intermediate precision-2	1094.72	101.36	
Intermediate precision-3	1104.87	102.30	
Intermediate precision-4	1075.15	99.55	
Intermediate precision-5	1070.13	99.09	
Intermediate precision-6	1093.12	101.21	
Mean		**100.08**	
StdDev		**1.11**	
% RSD		**1.11**	**NMT 2.0 %**

Discussion for Intermediate precision:

System suitability result passes and the results obtained for Intermediate precision are found within the acceptance criteria with reference to the table no. 8.17 to 8.21. Combined Intermediate precision results and method precision results are also found well within the acceptance criteria. The chromatograms showed in the chromatogram no. 7.52 to 7.65.

ROBUSTNESS

Table No: 8.22 System suitability results for Artemether

S. No.	Parameter Name	Artemether Results obtained		
		Tailing factor	Area (RSD)	Theoretical Plates
01	Change in wavelength Plus 212nm	1.05	0.051	9382
02	Change in wavelength Minus 208 nm	1.04	0.051	9385
03	Change in Flow rate 2.20 mL	1.03	0.087	9520
04	Change in Flow rate 1.80 mL	1.05	0.178	8924
05	Change in Mobile phase plus	1.02	0.165	9068
06	Change in Mobile phase minus	1.1	0.184	9057
Acceptance criteria		**NMT 2.0**	**NMT 2.0%**	**NLT 1000**

Table No: 8.23 System suitability results for Lumefantrine

S. No.	Parameter Name	Lumefantrine Results obtained			
		Tailing factor	**Area (RSD)**	**Theoretical Plates**	**Resolution**
01	Change in wavelength Plus 212nm	2.33	0.096	3838	16.58
02	Change in wavelength Minus 208 nm	2.23	0.102	3841	16.57
03	Change in Flow rate 2.20 mL	2.33	0.045	3610	16.52
04	Change in Flow rate 1.80 mL	2.31	0.124	4046	17.00
05	Change in Mobile phase plus	2.20	0.146	4155	17.09
06	Change in Mobile phase minus	2.20	0.070	4152	17.05
Acceptance criteria		**NMT 3.0**	**NMT 2.0%**	**NLT 1000**	**NLT 5.0**

Table No: 8.24 Robustness results obtained for Artemether

S. No.	Parameter Name	Results obtained		Acceptance criteria
		Artemether drug in mg	Artemether drug in %	
01	Robust Wavelength 212 nm	**180.32**	**100.18**	**90.0% - 110.0%**
02	Robust Wavelength 208 nm	**179.56**	**99.76**	
03	Robust flow rate 2.2 mL	**178.32**	**99.07**	
04	Robust flow rate 1.8 mL	**181.25**	**100.71**	
05	Robust mobile phase composition +5 %	**178.69**	**99.27**	
06	Robust mobile phase composition -5 %	**179.92**	**99.96**	

Table No: 8.25 Robustness results obtained for Lumefantrine

S. No.	Parameter Name	Results obtained		Acceptance criteria
		Lumefantrine drug in mg	Lumefantrine drug in %	
01	Robust Wavelength 212 nm	**1085.56**	**100.49**	**90.0% - 110.0%**
02	Robust Wavelength 208 nm	**1083.24**	**100.30**	
03	Robust flow rate 2.2 mL	**1084.54**	**100.42**	
04	Robust flow rate 1.8 mL	**1078.69**	**99.88**	
05	Robust mobile phase composition +5%	**1079.25**	**99.93**	
06	Robust mobile phase composition -5%	**1086.23**	**100.58**	

Table No: 8.26 Combined Method precision and Robustness results obtained for Artemether

S. No.	Parameter Name	Results obtained		
		Artemether drug in mg	**Artemether drug in %**	**Acceptance criteria**
01	Method precision - 1	**180.43**	**100.24**	**90.0%-110.0%**
02	Method precision - 2	**178.77**	**99.32**	
03	Method precision - 3	**176.98**	**98.32**	
04	Method precision - 4	**181.56**	**100.87**	
05	Method precision - 5	**183.46**	**101.92**	
06	Method precision - 6	**180.45**	**100.25**	
07	Robust Wavelength 212 nm	**180.32**	**100.18**	
08	Robust Wavelength 208 nm	**179.56**	**99.76**	
09	Robust flow rate 2.2 mL	**178.32**	**99.07**	
10	Robust flow rate 1.8 mL	**181.25**	**100.71**	
11	Robust mobile phase composition +5 %	**178.69**	**99.27**	
12	Robust mobile phase composition -5%	**179.92**	**99.96**	
Mean			99.99	
Std Dev			0.9458	**NMT 2.0%**
% RSD			0.9459	

Table No: 8.27 Combined Method Precision and Robustness Results Obtained for Lumefantrine

S. No.	Parameter Name	Results obtained		
		Lumefantrine drug in mg	**Lumefantrine drug in %**	**Acceptance criteria**
01	Method precision – 1	**1087.86**	**100.73**	**90.0% - 110.0%**
02	Method precision – 2	**1073.71**	**99.42**	
03	Method precision – 3	**1066.51**	**98.75**	
04	Method precision – 4	**1079.18**	**99.92**	
05	Method precision – 5	**1081.66**	**100.15**	
06	Method precision – 6	**1075.60**	**99.59**	
07	Robust Wavelength 212 nm	**1085.56**	**100.49**	
08	Robust Wavelength 208 nm	**1083.24**	**100.30**	
09	Robust flow rate 1.8 mL	**1084.54**	**100.42**	
10	Robust flow rate 2.2 mL	**1078.69**	**99.88**	
11	Robust mobile phase composition +5 %	**1079.25**	**99.93**	
12	Robust mobile phase composition -5 %	**1086.23**	**100.58**	
Mean			100.01	
Std Dev			0.5632	**NMT 2.0 %**
% RSD			0.5631	

Discussion for Robustness

The results obtained for Robustness are found within the acceptance criteria with reference to the table no. 8.22 to 8.27. Combined method precision and robustness results are also found well within the acceptance criteria. The chromatograms showed in the chromatogram no. 7.66 to 7.89.

STABILITY STUDIES

Table No. 8.28 System suitability results and Cumulative % RSD results obtained for Stability of standard solution (Artemether)

S. No.	Time point	Standard solution area	Results obtained		
			Cumulative % RSD	Tailing factor obtained	Theoretical plate obtained
01	0 hour	289180	NA	1.01	9382
02	3rd hour	289290	0.027	1.04	9387
03	6th hour	289395	0.037	1.01	9401
04	9th hour	289178	0.036	1.05	9378
05	12th hour	288905	0.063	1.03	9409
06	15th hour	289879	0.112	1.05	9415
07	18th hour	288956	0.112	1.06	9317
08	21st hour	288792	0.118	1.01	9358
09	24th hour	289417	0.114	1.04	9417
Limit			**NMT 2.0 %**	**NMT 2.0**	**NLT 1000**

Table No: 8.29 System suitability results and Cumulative % RSD results obtained for Stability of sample solution (Artemether)

S. No.	Time point	Sample solution area	Results obtained	
			Cumulative % RSD	Tailing factor obtained
01	0 hour	286899	NA	1.01
02	3rd hour	286597	0.074	1.06
03	6th hour	286456	0.079	1.04
04	9th hour	286954	0.083	1.05
05	12th hour	286546	0.078	1.06
06	15th hour	286785	0.071	1.01
07	18th hour	286546	0.068	1.03
08	21st hour	286379	0.073	1.06
09	24th hour	286347	0.077	1.03
Limit			**NMT 2.0 %**	**NMT 2.0**

Table No: 8.30 System suitability results and Cumulative % RSD results obtained for Stability of standard solution (Lumefantrine)

S. No.	Time point	Standard solution area	Results obtained			
			Cumulative % RSD	Tailing factor obtained	Theoretical plate obtained	Resolution
01	0 hour	4972130	NA	2.19	4165	17.09
02	3rd hour	4972651	0.007	2.18	4398	17.10
03	6th hour	4973256	0.011	2.2	4157	17.09
04	9th hour	4974785	0.023	2.17	4178	17.11
05	12th hour	4972256	0.022	2.2	4165	17.10
06	15th hour	4973125	0.019	2.19	4145	17.09
07	18th hour	4977845	0.041	2.20	4105	17.02
08	21st hour	4964528	0.075	2.19	4018	17.09
09	24th hour	4977136	0.077	2.18	4187	17.09
Limit			**NMT 2.0 %**	**NMT 3.0**	**NLT 1000**	**NLT 3.0**

Table No: 8.31 System suitability results and Cumulative % RSD results obtained for Stability of sample solution (Lumefantrine)

S. No.	Time point	Sample solution area	Results obtained	
			Cumulative % RSD	Tailing factor obtained
01	0 hour	4871372	NA	1.96
02	3rd hour	4892325	0.303	1.99
03	6th hour	4852837	0.405	1.97
04	9th hour	4902014	0.450	1.96
05	12th hour	4910045	0.479	1.95
06	15th hour	4899074	0.442	1.95
07	18th hour	4874021	0.418	1.97
08	21st hour	4872836	0.399	1.99
09	24th hour	4882235	0.373	1.99
Limit			**NMT 2.0 %**	**NMT 3.0**

Discussion for stability of analytical solutions:

System suitability result passes with reference to the table no. 8.28 to 8.31 and the results obtained for stability of standard solution and sample solution are found within the acceptance criteria for the minimum period of 24 hours study. The chromatograms were showed in the chromatogram no.7.90 to 7.116.

9. CONCLUSION

A RP-HPLC method for Artemether and Lumefantrine were developed and validated in powder for oral suspension dosage form as per ICH guidelines. The results are found to be complying with the acceptance criteria for each of the parameter.

Acetonitrile and phosphate buffer of pH 2.3 in the ration of 45:55 v/v was used as mobile phase. The detection was carried out at 210 nm. The column was Zorbax SB C_8 (250 x 4.6mm, 5µ). The flow rate was selected as 2mL/min

Agilent HPLC (Open Lab software with DAD detector) with Zorbax SB C_8 (150X 4.6mm, 5µ) Packed Column, Injection volume of 20µL was injected and eluted with the Mobile phase (acetonitrile and phosphate buffer of pH 2.3 in the ration of 45:55 v/v.) Which was pumped at a flow rate of 2.0 ml/min at 210nm.The peak of Artemether and Lumefantrine was found well separated at 4.3min, 12.3 min. The developed method was validated for various parameters as per ICH guidelines like system suitability, linearity, accuracy, precision, specificity, limit of detection, limit of quantitation, ruggedness, and robustness. The result obtained for the parameters were found well within the acceptance criteria.

Hence it is concluded that the assay method is found to be valid in terms of precision, accuracy and specificity and hence it is suitable for routine analysis as well as for stability analysis.

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